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| 13. ABSTRACT (Maximum 200 Words) All-trans retinoic acids (RA) inhibits proliferation of breast cancer cells. This effect is presumably mediated by the retinoic acid receptors (RAR). In the absence of RA, the RAR represses basal transcription through interaction with nuclear receptor corepressors such as SMRT (silencing mediator for retinoid and thyroid receptors) or N-CoR (nuclear receptor corepressor). In this project, we have characterized receptor interaction and transcriptional repression function of SMRT and N-CoR. We have investigated the expression and regulation of SMRT in breast cancer cells. Our data suggest that there are two independent nuclear receptor interacting domains and multiple repression domains on the corepressors. The hormone-binding domain of the receptor is involved in a ligand-independent interaction with the corepressors and it contains a C-terminus AF-2 helix that is essential for ligand-dependent dissociation of the corepressor. We have demonstrated that overexpression of SMRT enhances transcriptional repression of nature RA-responsive promoters. In addition, a novel SMRT isoform termed SMRTe has been isolated. SMRTe is very similar in structure and function to N-CoR. Analysis of SMRTe expression in breast cancer cells suggests that cancerous cells contain higher levels of SMRTe than normal breast cells. Taken together, these results suggest that SMRTe may be involved in regulation of breast cancer cell growth and proliferation. | | | | |
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Introduction

All-trans retinoic acid (RA) is known to inhibit growth of estrogen receptor (ER)-positive human breast carcinoma (HBC) cells (10, 11, 13). The action of RA is mediated by its intracellular nuclear receptor known as retinoic acid receptors (RAR), which belongs to the steroid/nuclear receptor superfamily (9). Nuclear receptors are DNA-binding proteins and their activities regulated by hormones. It is known that 17 β -estradiol (E2) can promote the growth of ER-positive HBC cells, while 4-hydroxytamoxifen (HTM) functions as an anti-estrogen, which enhances RA-induced growth arrest of HBC cells (1, 2, 6, 7, 12). The use of RA or its synthetic analogs as potential therapeutic agents in treating breast cancer is promising. However, how RA inhibits the growth of HBC cells, and how it interacts with E2 is currently unclear. We are interested in understanding the mechanisms of inhibition of RA on HBC cells and the cross-talk between RA and E2. Recently, several nuclear receptor associated proteins have been reported (4, 8). These proteins function as cofactors that help the receptors to activate and to repress target gene expression. The nuclear receptor corepressor SMRT (silencing mediator for retinoid and thyroid hormone action) and N-CoR (nuclear receptor corepressor) function as transcriptional corepressors that promote the repressor activity of unliganded RAR (3, 5). Since RAR plays an important role in the regulation of HBC cell proliferation, it is possible that regulation of RAR activity by corepressor is important in controlling breast cancer cell proliferation. We proposed a model to explain the cross-talk between RA and E2 (Figure 1). In this project, we have investigated the role of the corepressor in breast cancer through characterizing receptor interaction and transcriptional repression domains of SMRT and analyzing its expression in breast cancer cells.

Body

Objective 1. To analyze expression and regulation of SMRT in breast cancer cells

Task 1: Collecting breast cancer cell lines and isolation of total cellular RNA.

We have obtained and established several breast cancer cell lines in the laboratory. These include several ER-positive cells (T-47D, MCF-7M, MDA-MB-361, BT474 and MDA-MB-134) and ER-negative cells (MDA-MB-231, MDA-MB-330, BT 20, Hs0578T, MDA-MB-453). The normal breast epithelial cell line HBL100 is also established. These cell lines are maintained in Dulbecco's modified Eagle's medium (DMEM) and stocks are in liquid nitrogen. Total RNAs were isolated from these cells. Aliquots of these RNAs are stored in a -70°C freezer.

Task 2: Northern blot analysis of the expression levels of SMRT in different breast cancer cells.

We have conducted Northern blot analysis for the expression of SMRT in breast cancer cells. The SMRT probe was prepared by random priming reaction. Northern blot analysis showed that SMRT expressed as a 9 kb band at relatively low level in breast cancer cells. There were no obvious difference in the expression levels of SMRT in different cancer cell line. We also measured the protein level of SMRT by Western blotting using an anti-SMRT polyclonal

antibody. We detected the expression of SMRT in all breast cancer cells analyzed. Interestingly, we found that the level of SMRT in HBL100 cells is relatively less than in cancer cells (Figure 2). Furthermore, we found that the size of the major SMRT protein is 270-kDa, which is close to the size of N-CoR and is much larger than the expected SMRT protein. Western blot confirmed that the SMRT antibody did not cross-react with N-CoR (Figure 4).

Task 3: Treatment of breast cancer cells with hormones, isolation of RNA after treatment, and Northern blot analysis of the expression levels of SMRT.

We tested the expression of SMRT in HBC cells by Northern and Western blot and found that SMRT expression did not change significantly after hormone treatment. These results suggest that SMRT expression is not hormone-dependent. It is possible that protein-protein interaction between SMRT and nuclear receptors may play a role in the cross-talk between retinoic acid and estrogen.

Task 4: Isolation of SMRT genomic clones and identification of potential response elements.

Because the effect of hormone on the expression level of SMRT was not obvious, we decided not to continue studying regulation of SMRT gene expression.

Objectives 2: To investigate the involvement of nuclear receptor corepressors in cross-talk between retinoids and steroid hormones.

Task 5: Evaluating the protein-protein interaction between nuclear receptor corepressors SMRT and steroid receptors including ER, PR, GR and AR in vitro.

Using Far-Western assay, we detected an interaction between ER and SMRT (Figure 3). Interestingly, such association was enhanced slightly by E2 treatment (Figure 3). We tested the effect of anti-estrogen Tamoxifen on the interaction and found that Tamoxifen also slightly enhanced the interaction between SMRT and ER, similar to the effect of E2. However, because SMRT also interacts with many other nuclear receptors including RXR α in a manner that is much weaker than its interaction with RAR α or TR β , we speculated that the interaction with ER α might not be physiologically significant. This speculation was supported by the fact that overexpression of SMRT did not have an obvious effect on the ligand-dependent transcriptional activity of ER α (unpublished data).

We then further characterized the protein-protein interaction between SMRT and RAR α and TR β and details of these studies have been reported in a publication shown in the appendix (Molecular Endocrinology, 11: 2025-2037). Briefly, we found that SMRT interacted with RAR α and TR β very strongly in vitro and in vivo. Such interactions are sensitive to hormone treatment, presumably due to conformational change of the receptor upon ligand binding.

Task 6: Investigating the effects of hormone and anti-hormone binding on the protein-protein interaction between steroid hormone receptors and corepressors SMRT.

Except for the weak interaction between SMRT and ER α , we observed no interaction between SMRT and other steroid hormone receptors in the presence or absence of hormone or anti-hormone.

Task 7: Analyzing the protein complex of receptors and corepressors in breast cancer cells.

Because SMRT did not appear to interact strongly with ER, we decided not to analyze the protein complex of ER and the corepressors in breast cancer cells.

Key Research Accomplishments:

- Breast cancer cells express SMRT (the silencing mediator for retinoid and thyroid hormone action) at higher levels than normal breast epithelial cells.
- Two regions of thyroid receptor β (TR β) and retinoic acid receptor α (RAR α) are essential for interaction with SMRT.
- Two SMRT interaction domains for RAR α and TR β defined.
- Multiple transcriptional repression domains in SMRT identified.
- An extended form of SMRT termed SMRTe was identified. SMRTe is the major form of SMRT present in normal and cancerous breast epithelial cells.

Reportable outcomes:

- One article was published in "Molecular Endocrinology" (see Appendices 1)
- One review paper published in "Critical Rev. in Eukaryotic Gene Exp." (see Appendices 2)
- One book chapter is in-press in "Vitamins and Hormones" (see Appendices 3).
- A funding applied to NIH based on work supported by this award.

Conclusions:

In summary, we have analyzed the expression of SMRT in breast cancer cells. We found that breast cancer cells express higher level of SMRT than normal breast epithelial cells. We have also characterized the receptor interacting surfaces on SMRT and RAR α and TR α . Furthermore, we have characterized the transcriptional repression function of SMRT and found that SMRT contains multiple repressor domains, which interact with other corepressor proteins. Most interestingly, we have identified an extended form of SMRT (SMRTe) which appears to be the major product of the SMRT gene.

Because SMRT does not seem to interact with ER or other steroid hormone receptor strongly, it is likely that the effect of SMRT on the cross-talk between RA and E2 is due to modulation of RAR α activity by SMRT. Furthermore, the identification of SMRTe suggests that it may be more important to study SMRTe in breast cancer. Therefore, future studies will focus on understanding the role of SMRTe in the cross-talk between RA and E2 in breast cancer cells.

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Appendices:

Figure 1. Model of SMRT action in breast cancer.

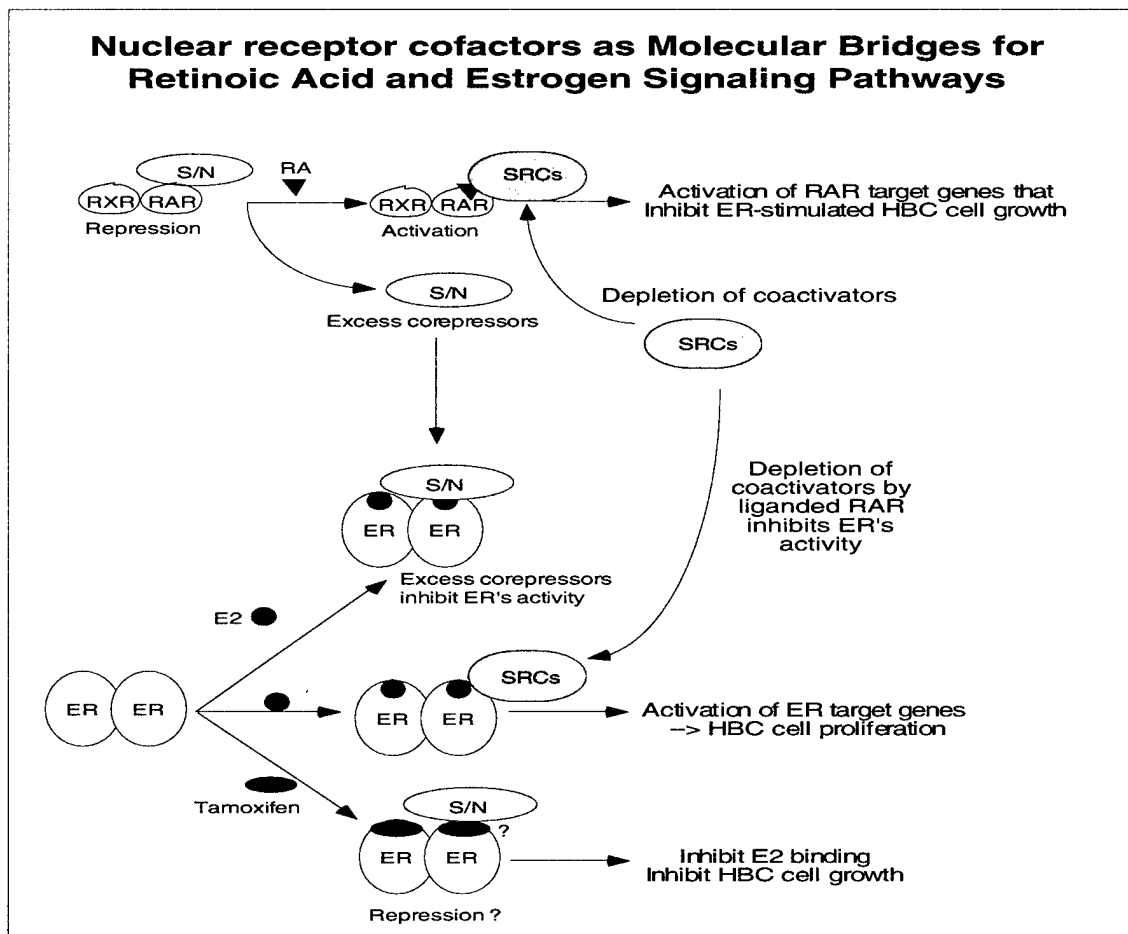


Figure 2. SMRT expression in breast cancer cells. Total cell extracts were separated on a SDS-PAGE, blotted onto a nitrocellulose filter and hybridized with an affinity purified anti-SMRT antibody on total cell extract. The apparent molecular weight of the SMRT signal is 270-kDa (also see Figure 4).

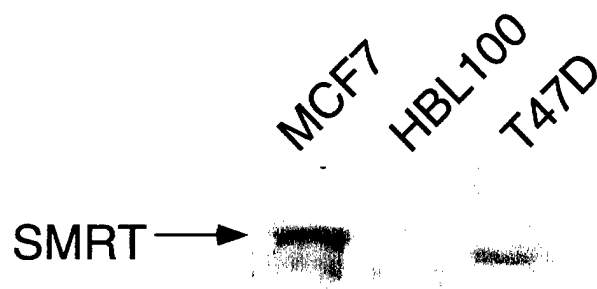


Figure 3. SMRT interacts with ER. We blotted the purified GST-C-SMRT on nitrocellulose filters and hybridized with ^{35}S -methionine labeled nuclear receptors in the absence or presence of hormones. We used *all-trans* RA for RAR, 9-*cis* RA for RXR, T3 for TR and 17 β -estradiol for ER at 1 μM concentration). The last lane shows the GST-SMRT fusion protein on the gel after staining by commassie blue. These results indicate that SMRT are not only capable of interacting with unliganded RAR and TR, but also capable of interacting with ER.

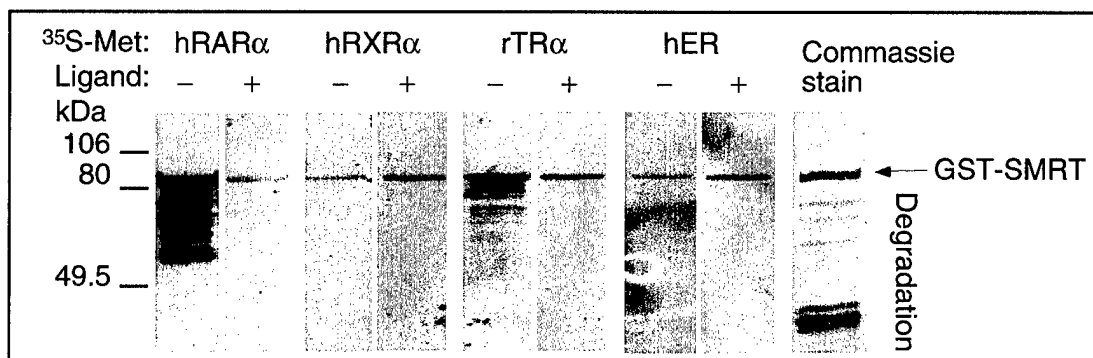
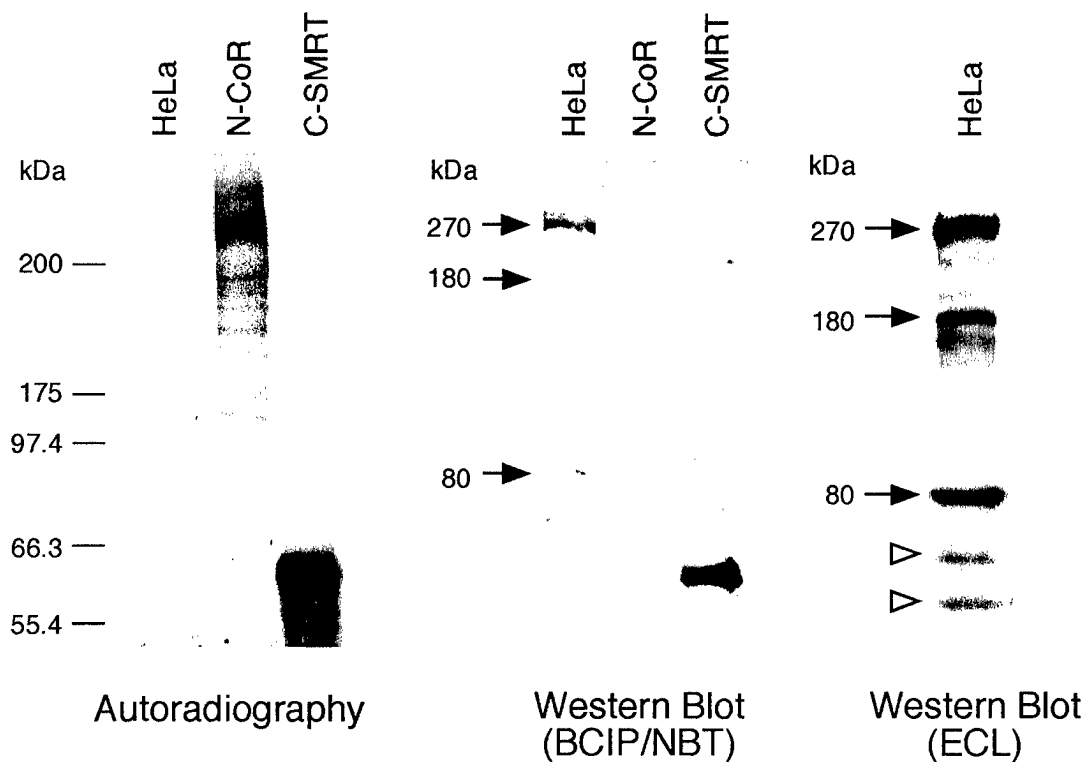


Figure 4. Identification of an extended form of SMRT. HeLa nuclear extract, together with in vitro-translated ^{35}S -methionine labeled N-CoR and C-SMRT, were separated on a SDS-PAGE. The N-CoR and C-SMRT polypeptides were detected by autoradiography (left). An identical gel was processed for Western blotting using an affinity purified rabbit anti-C-SMRT polyclonal antibody and detected by BCIP/NBT color reaction (center). One major polypeptide similar to the size of N-CoR (270-kDa) was detected in the HeLa nuclear extract, in addition to two minor bands of 180 and 80-kDa, respectively (arrows). The anti-SMRT antibody does not cross-react with N-CoR. The same HeLa nuclear extract was also processed for Western blotting using anti-C-SMRT antibody but developed by ECL⁺ reaction (right). The three specific SMRT polypeptides and two non-specific bands (open arrowheads) below 80-kDa were indicated.



Bibliography of all publications and meeting abstracts

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Characterization of Receptor Interaction and Transcriptional Repression by the Corepressor SMRT

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SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) and **N-CoR** (nuclear receptor corepressor) are two related transcriptional corepressors that contain separable domains capable of interacting with unliganded nuclear receptors and repressing basal transcription. To decipher the mechanisms of receptor interaction and transcriptional repression by SMRT/N-CoR, we have characterized protein-protein interacting surfaces between SMRT and nuclear receptors and defined transcriptional repression domains of both SMRT and N-CoR. Deletional analysis reveals two individual nuclear receptor domains necessary for stable association with SMRT and a C-terminal helix essential for corepressor dissociation. Coordinately, two SMRT domains are found to interact independently with the receptors. Functional analysis reveals that SMRT contains two distinct repression domains, and the corresponding regions in N-CoR also repress basal transcription. Both repression domains in SMRT and N-CoR interact weakly with mSin3A, which in turn associates with a histone deacetylase HDAC1 in a mammalian two-hybrid assay. Far-Western analysis demonstrates a direct protein-protein interaction between two N-CoR repression domains with mSin3A. Finally we demonstrate that overexpression of full-length SMRT further represses basal transcription from natural promoters. Together, these results support a role of SMRT/N-CoR in corepression through the utilization of multiple mechanisms for receptor interactions and transcriptional repression. (*Molecular Endocrinology* 11: 2025-2037, 1997)

INTRODUCTION

Transcriptional regulation by steroid/thyroid hormones and retinoids is a critical component in controlling many aspects of animal development, reproduction,

and metabolism (1-4). The functions of these hormones are mediated by intracellular receptors, which comprise a large superfamily of ligand-dependent transcription factors (1). It has been established that both retinoic acid receptors (RARs) and thyroid hormone receptors (TRs) function via formation of heterodimeric complexes with retinoid X receptors (RXRs) (5, 6). Once bound to a DNA response element, the heterodimer responds to ligand through the C-terminal ligand-binding domain (LBD), which is known to mediate not only hormone binding but also receptor dimerization, transcriptional activation, and repression (7, 8).

Both TR and RAR can function as transcriptional repressors in the absence of ligands and potent activators upon binding of ligands (7). DNA-binding assays and functional analysis have demonstrated that the repressor activities of unliganded receptors depend on DNA response elements, as well as on the intact LBD of the receptors (7, 9, 10). *In vivo*, the TR/RXR heterodimer binds to DNA in the context of chromatin, and nucleosome assembly enhances the transcriptional silencing effect (11). Importantly, the oncogenic activity of v-erbA, a mutated form of TR, is directly linked to transcriptional repression (12, 13). In addition, deletion of the activation domain of RAR converts it into a potent transcriptional repressor, and this mutation was shown to cause defects in cellular differentiation and development (14-16). Therefore, transcriptional repression by unliganded nuclear receptors appears to play an important role in regulating cell growth and differentiation.

Hormone binding is thought to induce conformational changes that lead to ligand-dependent transformation of the receptors from repressors to activators (1). The C terminus of TR, about 20 amino acids, constitutes the 12th amphipathic helix (helix 12) of the LBD (17-19), which functions as a ligand-dependent activation core domain known as the AF2-AD, α C, or α 4 domain (8, 20-22). Comparison of the LBD structures of the unliganded (19) and liganded receptors (17, 18) reveals a striking difference in the relative position of the helix 12/AF2-AD domain. This posi-

tional shift is thought to play an important role in receptor activation, allowing the liganded receptors to displace corepressors (8, 23–25) and to interact with coactivators (see reviews in Refs. 26–28).

SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) and N-CoR (nuclear receptor corepressor) are two related transcriptional corepressors (24, 25) that are distinct from other proteins (29). They were shown to utilize the C-terminal domain for interaction with unliganded receptors (30–33), and the N-terminal domain for transcriptional repression (25, 30). In this study, we investigate mechanisms of protein-protein interactions between SMRT and nuclear receptors and analyze the modes of repression mediated by SMRT/N-CoR. To do this, we define the interacting surfaces between SMRT and nuclear receptors in binding and functional assays. Next, we compare transcriptional repression mediated by SMRT and N-CoR using transient transfection assays in mammalian cells. Evidence is presented that SMRT and N-CoR interact with additional corepressors, and that histone deacetylation plays a role in SMRT/N-CoR-mediated repression.

RESULTS

Two Receptor Domains Are Essential for Interaction with SMRT

Deletion mutants in the carboxyl and amino termini of TR and RAR were used to analyze the contribution of different regions in the receptors for protein-protein interaction with SMRT. Figure 1A shows the domain structure of TR and the relative position of individual helices in the LBD as determined by x-ray crystallography (17, 18). The sequence at the C terminus region around helices 11 and 12 is also shown for both TR and RAR. [³⁵S]Methionine-labeled TR or RAR deletion mutants were hybridized to glutathione S-transferase (GST)-SMRT and GST-RXR in far-Western analyses in the absence of hormone (Fig. 1B). The relative strengths of these interactions are summarized in Fig. 1C.

Full-length TR (1–456) associates well with both SMRT and RXR, and the interaction with SMRT can be drastically reduced upon hormone treatment. A residual weak interaction was observed in the presence of ligand, consistent with previous observations (24, 30). Carboxyl-terminal truncation at residue 441, which deletes helix 12, results in a mutant that interacts normally with RXR but that exhibits enhanced interaction with SMRT. Further truncation at residue 423, which removes part of helix 11, reduces the interaction with SMRT back to a level similar to that of wild type TR. In contrast, this deletion markedly reduces interaction with RXR. Further deletions that remove additional helices (helices 8, 9, and 10) result in barely detectable interaction with SMRT and no interaction with RXR. These results suggest that helix

12 inhibits SMRT association while helix 11 might promote the association.

Amino-terminal truncation of TR at residue 173, which removes the DNA-binding domain (DBD), does not affect the interaction with either SMRT or RXR. Further N-terminal deletion to residue 260, which removes the first and second helices of the TR LBD, markedly impairs SMRT association. No interaction with RXR by this mutant was detectable. Similarly, C-terminal deletion of helix 12 from RAR (1–403) also increases interaction with SMRT as compared with that of wild type RAR (1–462). Further deletion to residue 395, which removes part of helix 11, diminishes the enhanced interaction to a level comparable with that of full-length RAR, and ligand has little effect on the interaction. Together, these results identify two distinct interacting domains at the N-terminal hinge and C-terminal helix 11 regions of the receptor LBD that might act synergistically to promote interaction with SMRT. We find that the other two RAR isoforms, β and γ , also interact with SMRT in a ligand-reversible manner, although the interactions observed are weaker compared with that with RAR α (Fig. 1D). The interactions of both RAR β and RAR γ with RXR were not affected by ligand treatment.

Interaction of Helix 12/AF2-AD Deletion Mutants with SMRT in Yeast

To further understand the role of helix 12/AF2-AD in interaction with SMRT, we analyzed interactions between AF2-AD deletion mutants of RAR and RXR with C-terminal receptor-interacting domain of SMRT in a yeast two-hybrid system (Fig. 2). The RAR LBD alone is sufficient to interact with SMRT in a ligand-reversible manner (Fig. 2A, column 3), but the resulting activity is much weaker compared with that of full-length RAR (column 9). Similar to the far-Western results, SMRT and full-length RAR retain some interaction, even after treatment of the yeast cells with a saturating amount of ligand. It is unclear whether this observation reflects an association between liganded receptors and SMRT or the existence of a small percent of unliganded receptors after ligand treatment. Deletion of the AF2-AD domain results in a RAR mutant that stimulates gene expression in response to hormone treatment in yeast (columns 4 and 10), as opposed to the dominant negative activity of this mutant observed in mammalian cells (14). The ligand-dependent activation of RAR403 is more obvious in the context of full-length receptor (column 10). A similar effect has been shown in v-erbA, which normally acts as a constitutive repressor in mammalian cells, but as a ligand-dependent activator in yeast (34). Cotransformation of the RAR403 mutants with a Gal4 activation domain-SMRT fusion (Gal4 AD-SMRT) strongly induces β -galactosidase expression, even in the absence of hormone (columns 5 and 11). Furthermore, in contrast to the hormone-dependent dissociation seen with full-length RAR, hormone treatment does not interrupt

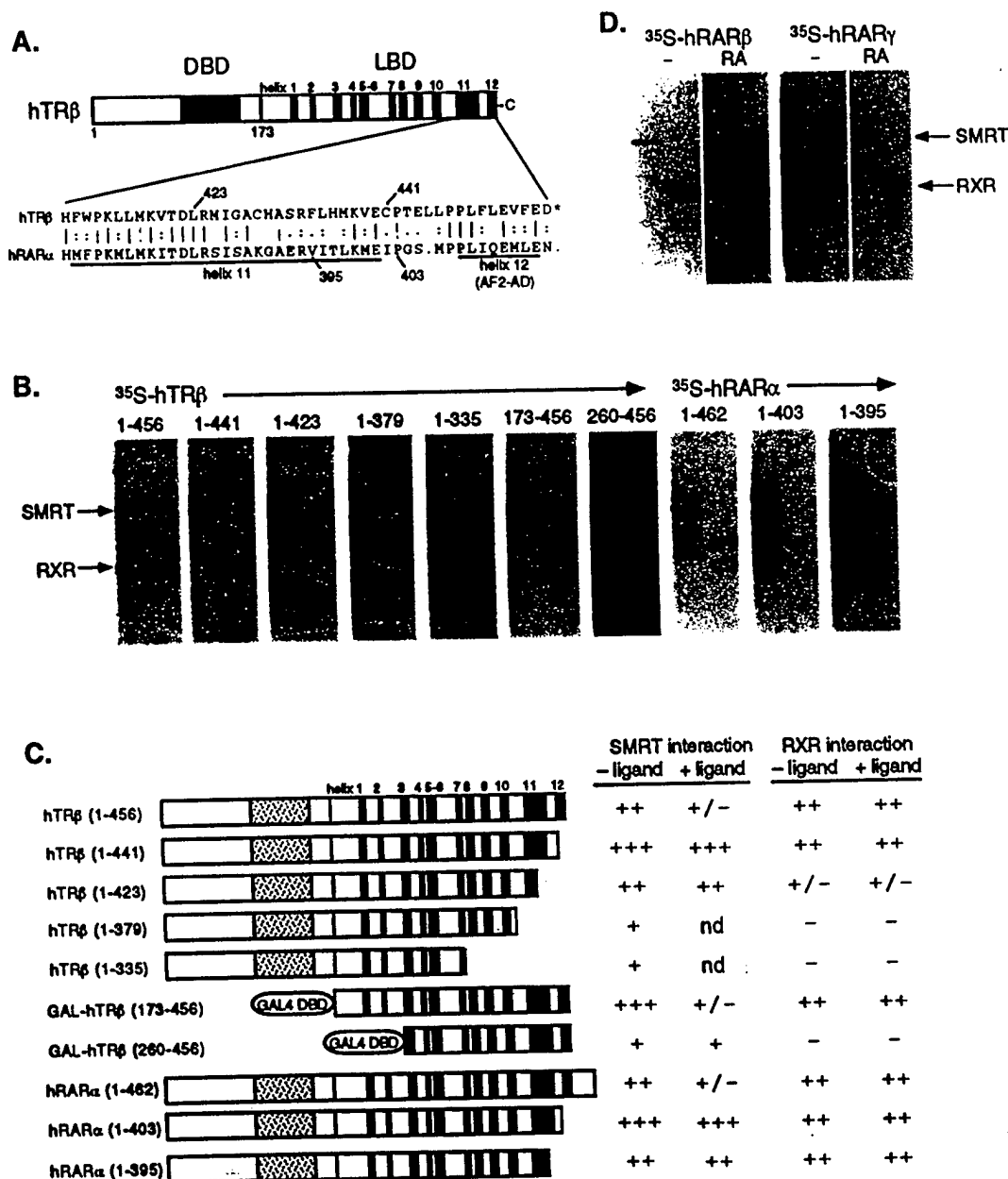


Fig. 1. Two Receptor Domains Interact with SMRT

A, Domain structure of human TR β and the sequences of the C-terminal helix 11 and 12 (AF2-AD) region of TR and RAR. The relative positions of individual helices determined by x-ray crystallography (18) are also indicated. B, Protein-protein interactions between receptors and SMRT or RXR in far-Western analyses. The full-length TR and RAR and their deletion derivatives were translated *in vitro* and labeled by [³⁵S]methionine. All these deletion mutants expressed similar amounts of proteins as analyzed by SDS-PAGE and autoradiography (not shown). The position of GST-C-SMRT (SMRT) and GST-RXR (RXR) fusion proteins are as indicated (arrows). Please note that GST-RXR appeared as a doublet in our extract. C, Summary of relative levels of interactions between receptor mutants and SMRT or RXR. The relative levels of interactions were scored from background level (-) to strong (+++). nd, Not done. D, Human RAR β and RAR γ interact with SMRT in a ligand-reversible manner in far-Western blots. -, vehicle only; RA, 1 μ M of all-trans-retinoic acid.

these interactions. Similarly, the Gal4 DBD-SMRT fusion interacts strongly with the Gal4 AD-RAR403 mutants in a ligand-insensitive manner (columns 6 and 12). These results are consistent with the enhanced interaction observed *in vitro* and indicate that the

AF2-AD domain may act as a negative regulatory element, controlling hormone-sensitive interaction between SMRT and nuclear receptors.

The effect of AF2-AD deletion in RXR on association with SMRT was also analyzed in the two-hybrid sys-

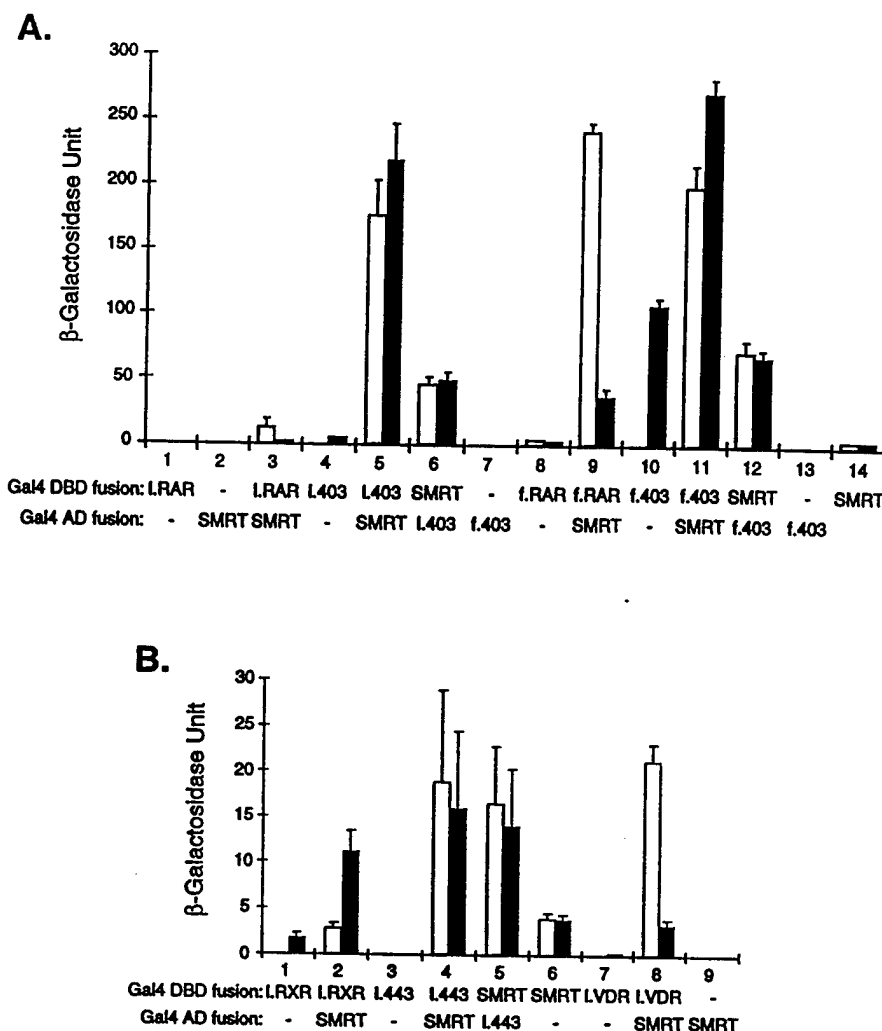


Fig. 2. Two-hybrid Interactions between SMRT and Helix 12/AF2-AD Deletion Mutants of Nuclear Receptors

A, Interaction between RAR403 and C-terminal domain of SMRT in yeast two-hybrid system. The indicated Gal4 AD and Gal4 DBD fusion constructs were cotransformed into yeast Y190 cells, and the resulting β -galactosidase activities were determined from three independent colonies. The β -galactosidase activities were determined in the absence (open bars) or presence (closed bars) of $1 \mu\text{M}$ of all-trans-RA. l, Ligand binding domain; f, full length; 403, RAR403 mutant with C-terminal truncation at residue 403. B, Interaction of SMRT with RXR443 and VDR in the absence of hormone (open bars) or presence (closed bars) of $1 \mu\text{M}$ 9-cis-RA (for RXR) or 100 nM 1,25 dihydroxyvitamin D_3 (for VDR). 443, RXR443 mutant with C-terminal truncation at residue 443.

tem (Fig. 2B). Ligand treatment weakly activates the Gal4 DBD-RXR LBD fusion (column 1), while cotransformation with Gal4 AD-SMRT enhances reporter gene expression (column 2), suggesting that SMRT can interact with RXR in either absence or presence of ligand. Truncation at residue 443 enhances the association between RXR and SMRT, and treatment with ligand does not alter this interaction (columns 4 and 5). These results suggest that SMRT can interact with RXR and that the AF2-AD domain of RXR also acts negatively in SMRT association. Furthermore, we observed a significant interaction between vitamin D_3 receptor (VDR) and SMRT in the absence of hormone, and treatment with ligand reduces the interaction (column 8). This result is consistent with the recent finding that VDR also contains intrinsic transcriptional repres-

sion activity (35), suggesting that SMRT might mediate transcriptional repression by VDR.

Two SMRT Domains Mediate Differential Interactions with Nuclear Receptors

The finding that two regions of TR are essential for protein-protein interaction with SMRT suggests that SMRT might also contain duplicated receptor-interacting domains. Several deletion mutants of SMRT were used to test this possibility in a far-Western blot, and the results are summarized in Fig. 3A. The GST fusions of these SMRT mutants were overexpressed, and the purified proteins (Fig. 3B, lanes 1 and 2) or crude extracts (lanes 3, 4, and 5) were analyzed for interaction with ^{35}S -labeled RAR and TR. SMRT(981-

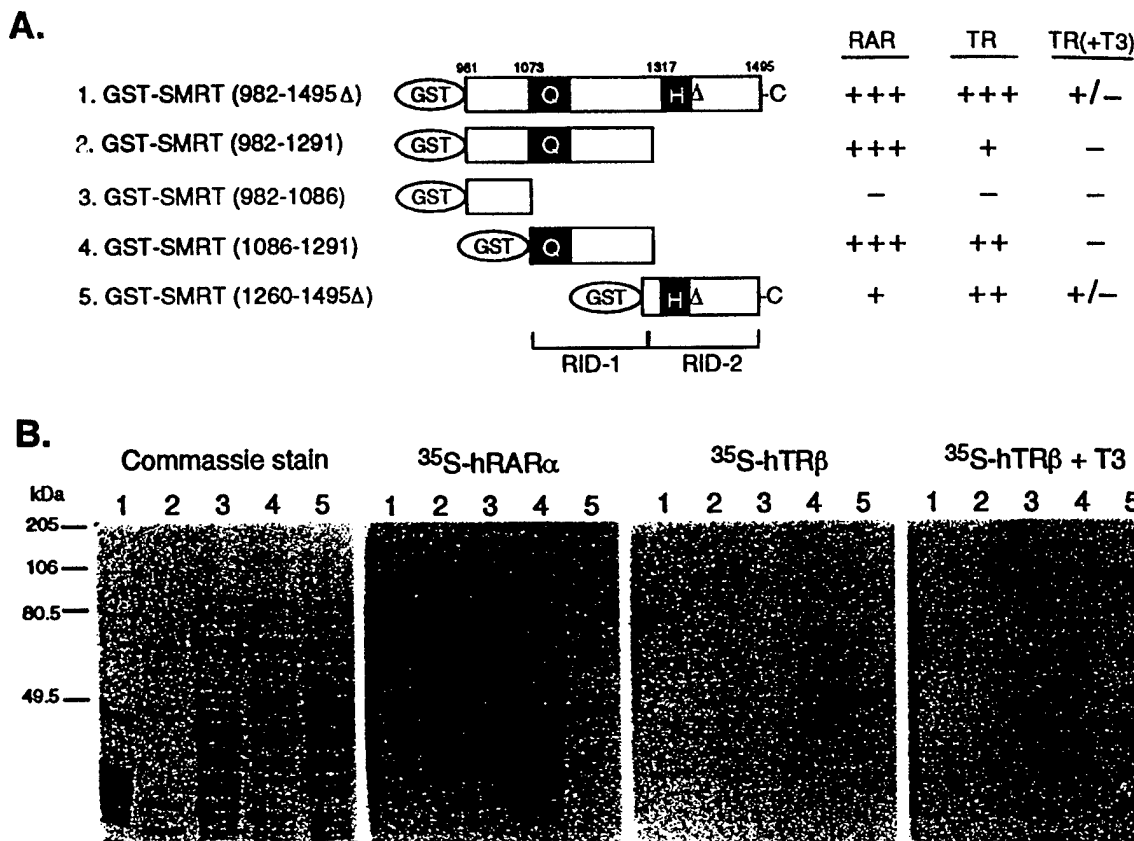


Fig. 3. Two SMRT Domains Interact with the Receptors

A, Summary of SMRT deletion mutants used in this experiment and their relative levels of interaction with nuclear receptors in far-Western analyses shown in panel B. The amino acids encoded by the SMRT mutants are shown in parentheses. Bound-RAR and TR were detected by autoradiography, and the relative levels of interaction were scored from background level (-) to strong (+++). The column numbers in each panel correspond to constructs shown in panel A. Partially purified GST fusion proteins were used in lanes 1 and 2 and total cell extracts were used in lanes 3, 4, and 5. RID, Receptor interacting domain. + T₃, Plus 1 μM T₃; Q, glutamine-rich domain; H, putative helical region; Δ, an internal deletion between amino acids 1330 and 1375 resulting from alternative splicing.

1495Δ) interacts equally well with both RAR and TR in the absence of ligands. RAR, but not TR, also interacts with degradation products of SMRT(981-1495Δ). Similarly, several fast migrating products of SMRT(1086-1291) also interact well with RAR, but not with TR (lane 4). These results indicate that RAR and TR may interact differently with SMRT. Consistent with this speculation, we find that SMRT(982-1291) (lane 2) as well as SMRT(1086-1291) interact more strongly with RAR than with TR. In contrast, the C-terminal fragment (1260-1495Δ) interacts better with TR than with RAR (lanes 5). All these interactions were found to be sensitive to hormone treatment (Fig. 3B and data not shown). Together, these results identify two independent receptor interacting domains (RID-1 and RID-2) of SMRT that appear to display different affinities to TR and RAR.

Two SMRT Repression Domains

In addition to the C-terminal receptor interacting domains, SMRT/N-CoR proteins also contain strong

transcriptional repression activity at their N-terminal regions. To define the minimal region needed for repression by SMRT, serial SMRT deletion mutants were generated, and their repression activities were analyzed using transient transfection (Fig. 4A). Consistent with previous observations, full-length as well as N-SMRT (amino acids 1-981) repress basal transcription strongly and in a dose-dependent fashion (rows 2 and 3), while C-SMRT (amino acids 982-1495Δ) exhibits minimal repression (row 4) compared with Gal4 DBD alone (row 1). Further deletion from the C terminus of N-SMRT reveals that amino acids 743 to 981 are not necessary for repression (row 5), while deletion to residue 475 reduces the repression effect about 2-fold (row 6). These results suggest that amino acids 475 to 981 may contribute in part to SMRT repression. Further C-terminal deletion to residue 337 drastically interferes with repression (row 7), indicating that the N-terminal boundary of this SMRT repression domain-1 (SRD-1) is located between amino acids 337

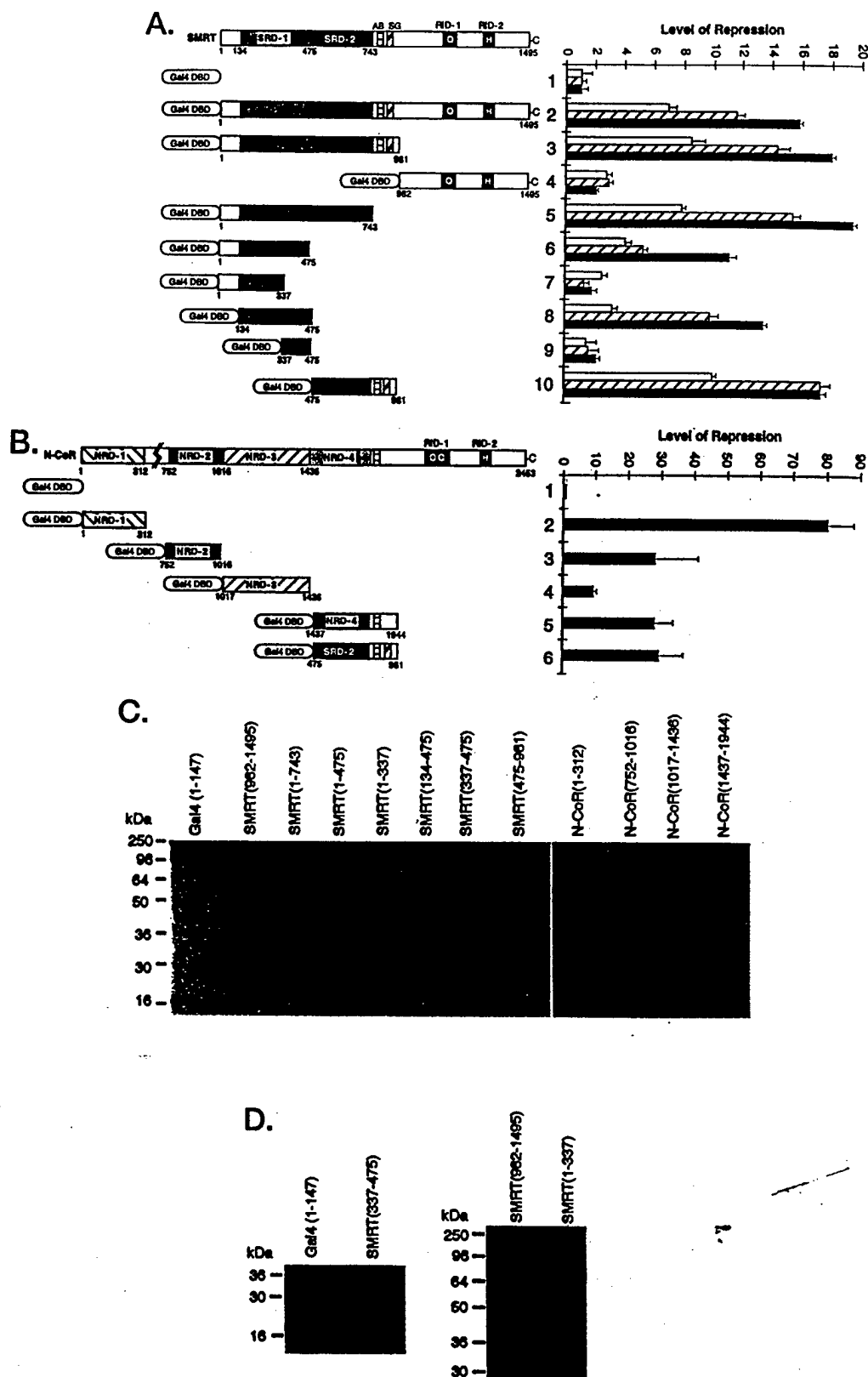


Fig. 4. Multiple Transcriptional Repression Domains

A, Deletion mapping of the repression domains of SMRT. The transcriptional repression activities were analyzed by transient transfection in CV-1 cells. The relative levels of repression were determined from an average of three independent transfections

and 475. Truncation from the N terminus reveals that amino acids 1–134 are dispensable for repression by SRD-1 (row 8), while further deletion to residue 337 abolishes repression (row 9), indicating that the C-terminal boundary of the SRD-1 is within amino acids 134–337. When the SMRT fragment between amino acids 475 and 981 was tested for repression, we found that this fragment also strongly repressed basal transcription (row 10). Together with the observation that amino acids 743–981 are not important for repression, these results may define amino acids 475–743 as a second, independent SMRT repression domain (SRD-2).

Sequence comparison between SMRT and N-CoR reveals that they share about 45% identity within both SRD-1 and SRD-2, suggesting potential functional conservation. Therefore, we tested whether the two SRD corresponding regions of N-CoR also contain repression activities. Consistent with a previous observation (25), amino acids 1–312 and 752–1016 of N-CoR exhibit strong repression activities (Fig. 4B, rows 2 and 3), and the two N-CoR domains corresponding to SRD-1 and SRD-2 also yield 10- to 30-fold repression (rows 4 and 5), similar to the repression effects observed by SRD-1 and SRD-2. These two additional N-CoR repression domains are termed N-CoR repression domain 3 and 4 (NRD-3 and NRD-4), and the two N-terminal repression domains are called NRD-1 and NRD-2. Together, these results indicate that both SMRT and N-CoR contain multiple, independent transcriptional repression domains.

To confirm that lack of repression in some of these SMRT/N-CoR deletion mutants is not due to lack of appropriate protein expression, we analyzed the expression of these constructs by both *in vitro* translation and Western blot analysis after transient transfection. We find that all constructs used in this experiment express approximately equal amounts of Gal4 DBD fusion proteins *in vitro* (Fig. 4C) and that the repression-defective mutants express well *in vivo* (Fig. 4D). These results indicate that lack of repression by certain SMRT/N-CoR deletion mutants are not due to lack of protein expression.

Multiple Mechanisms of Transcriptional Repression by SMRT/N-CoR

The mechanism of transcriptional activation by nuclear receptors has been shown to require recruitment of

coactivators, including histone acetyltransferases such as CBP/p300 (36–39). The opposite of histone acetylation, histone deacetylation, has recently been implicated in transcriptional repression by unliganded receptors and the associated corepressors. Several reports have described a corepressor complex containing a Mad-dependent corepressor mSin3A, a histone deacetylase HDAC1 or mRPD3, and the nuclear receptor corepressor SMRT/N-CoR (40–48). These results suggest that histone deacetylation may be a mechanism of transcriptional repression by unliganded receptors.

To confirm the interaction between mSin3A and the defined repression domains of SMRT and N-CoR, we tested the interactions between mSin3A and the individual repression domains of SMRT/N-CoR in a mammalian two-hybrid system. Coexpression of a VP16 AD-mSin3A fusion with all Gal4 DBD-SMRT/N-CoR repression domain fusions results in weak reduction of the repression activities (Fig. 5A). Coexpression of VP16 AD-mSin3A with a Gal4 DBD-HDAC1 fusion also results in partial release of repression mediated by Gal4 DBD-HDAC1 fusion. However, no activation above the background level was observed even though a VP16 activation domain was present. Since the weak interaction between SMRT/N-CoR repression domain with mSin3A in the two-hybrid system may reflect a dominant effect of repression over activation, we tested the interaction between mSin3A and individual SMRT/N-CoR repression domains *in vitro* by far-Western analysis. Full-length mSin3A was translated and labeled *in vitro* and used as a probe for GST fusions of various SRD and NRD domains. We find that mSin3A interacts specifically and consistently with NRD-1 and NRD-4 in this assay (Fig. 5B). In one experiment, we also detected interaction between SRD-2 and mSin3A (data not shown). No interaction is observed between SRD-1, NRD-2, and NRD-3. Therefore, these results suggest that different SMRT and N-CoR repression domains may repress transcription in a mSin3A-dependent or -independent manner.

SMRT Represses Basal Transcription from Natural Promoters

The hypothesis that SMRT/N-CoR proteins are transcriptional corepressors that facilitate repression by unliganded receptors is supported by protein-protein

using 0.1 μ g (open bars), 0.2 μ g (hatched bars), or 0.5 μ g (closed bars) of plasmid DNAs. The starting and ending amino acids in each deletion construct are shown beneath each domain. SRDs, SMRT repression domains. B, Deletion mapping of the N-CoR repression domains (NRDs). The N-CoR domains are aligned with those of SMRT in panel A. The relative levels of repression were determined using 0.5 μ g plasmid DNA and comparing the result to the Gal4 DBD alone. Two new transcriptional repression domains in N-CoR were found in addition to NRD-1 and NRD-2, which were identified previously (25). C, SDS-PAGE analysis of *in vitro* translated products of SMRT/N-CoR deletion constructs used in panels A and B. Two microliters of the *in vitro* translated products were analyzed in a 12.5% acrylamide gel, which was exposed overnight. Note that most of these constructs appear to produce doublet bands, perhaps due to secondary structure of the DNA used in the translation reaction. D, Western blot analysis of the repression-defective mutants of SMRT after transient transfection into 293 cells by using anti-Gal4 DBD monoclonal antibody (0.02 μ g/ml) and detected by ECL kit. The gel on the left was resolved in a 12.5% acrylamide gel while the gel on the right was resolved in a 10% gel.

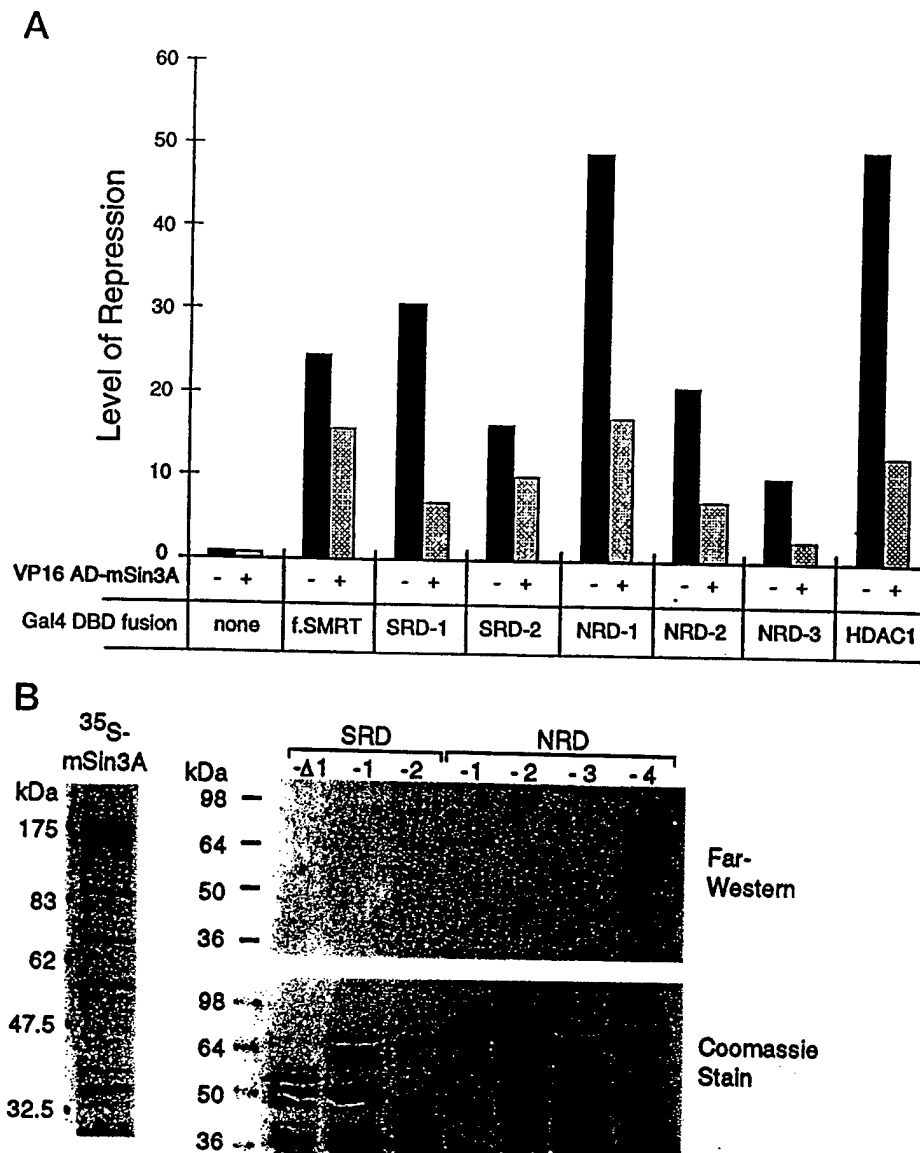


Fig. 5. Multiple Mechanisms of Transcriptional Repression by SMRT and N-CoR

A. Two-hybrid interactions of mSin3A with SMRT and N-CoR repression domains and HDAC1. The indicated Gal4 DBD fusion of SMRT and N-CoR repression domains and HDAC1 were transiently transfected into CV-1 cells together with either Gal4 AD alone or Gal4 AD-mSin3A fusion as indicated. The relative levels of repression are expressed as the means of three independent experiments relative to the Gal4 DBD alone. **B.** *In vitro* protein-protein interactions between mSin3A and SMRT/N-CoR repression domains. The GST fusions of various SRD and NRD domains were expressed in *Escherichia coli* and partially purified. The GST fusion proteins were analyzed by SDS-PAGE (right bottom panel) and examined for their abilities to interact with ³⁵S-labeled mSin3A in a far-Western blot (left upper panel). mSin3A appears to interact preferentially with intact GST-NRD-1 and GST-NRD-4 domains.

interactions and transient transfections using the Gal4 fusion system. To provide further evidence that SMRT may be physiologically relevant in transcriptional regulation, we tested the effect of SMRT overexpression on transcriptional activity of receptor-responsive promoters. Overexpression of full-length SMRT (Fig. 6, lane 2), but not that of C-SMRT lacking the repression domains (lane 3), repressed basal expression from a mouse RAR β 2 promoter approximately 2-fold in com-

parison to the empty vector (lane 1). The same result is evident with two minimal response elements in the context of a thymidine kinase promoter in the absence of hormone (Fig. 5A). As expected, hormone treatment enhanced transcription from these promoters, while overexpression of full-length SMRT reduced slightly this ligand-dependent activation. C-SMRT enhances the ligand-dependent activation from these promoters (Fig. 5B). These results suggest that SMRT may, at

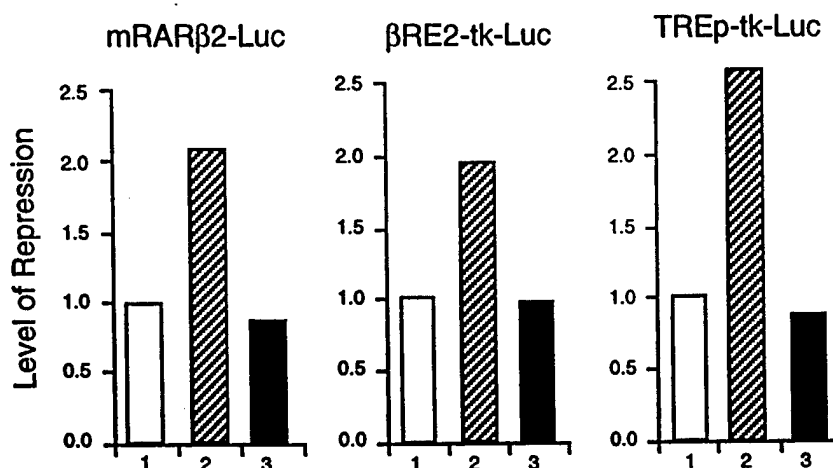
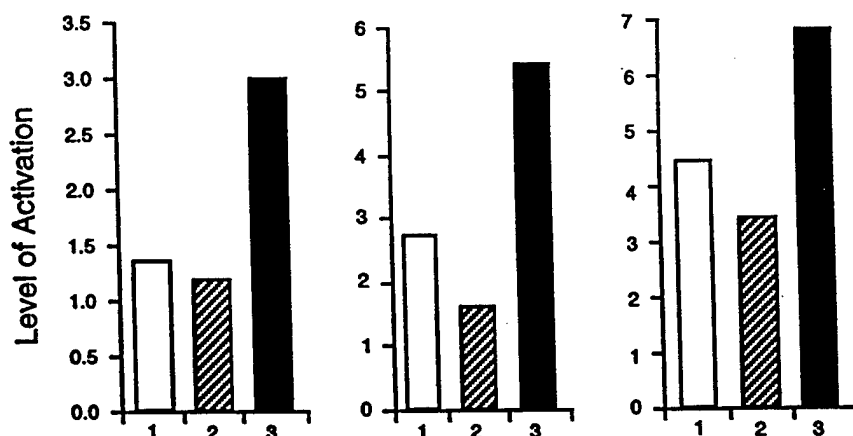
A. No hormone**B. 1 μ M atRA**

Fig. 6. SMRT Represses Basal Transcription from RAR- and TR-Responsive Promoters

The mRAR β 2 promoter, two copies of the β RARE (β RARE-tk-luc), and the TRE (TRE-tk-luc) response elements were linked to a luciferase reporter and transiently transfected into CV-1 cells together with empty vector alone (lanes 1), full-length SMRT expression vector (lanes 2), or C-SMRT expression vector (lanes 3). The relative level of repression in the absence of hormone is shown in panel A, while the relative level of activation in the presence of 1 μ M all-*trans* retinoic acid (atRA) is shown in panel B.

least under certain circumstances, facilitate transcriptional repression of natural promoters.

DISCUSSION

Transcriptional repression has been recognized as a critical component of TR and RAR function and is thought to be mediated by association of unliganded receptors with silencing mediators (corepressors) such as SMRT and N-CoR. To understand the function

of these putative corepressors, we have characterized their respective receptor interaction and transcriptional repression properties. Two distinct receptor-interacting domains of SMRT are identified that may interact directly with two corresponding regions in the receptor. We find that SMRT utilizes at least two distinct domains (SRD-1 and SRD-2) for transcriptional repression, consistent with a recent report (42). The two SRD-corresponding regions in N-CoR also repress basal transcription, indicating that N-CoR contains four independent repression domains. These results demonstrate the existence of multiple and

possibly redundant receptor interaction and transcriptional repression domains in SMRT and N-CoR. One might expect that this multiplicity will ensure a reliable targeting of the corepressors and appropriate repression of target genes before activation.

The hinge region of TR was originally shown to interact directly with the RID-2 region of N-CoR (25). Our results indicate that TR requires an additional C-terminal region for efficient association with SMRT. Nested deletion analyses suggest that helix 11 of the TR LBD plays an important role in stabilizing SMRT association, presumably by cooperating with the N-terminal helix 1-2 region. The interaction of SMRT with either the N terminus or C terminus of the LBD alone is very weak but detectable, suggesting that these two potential interacting surfaces may act synergistically to promote protein-protein interactions and to ensure appropriate recruitment of the corepressors. Similarly, two independent regions in the receptor have been shown to act synergistically for interaction with N-CoR (32, 49, 50). It has recently been shown that a receptor dimer is required for interaction with SMRT/N-CoR and that SMRT/N-CoR may contribute to receptor-specific transcriptional repression (51). Furthermore, an antagonist of the transcriptional activation by RXR homodimer was shown to promote association with the corepressor SMRT (52). Together, these studies suggest that SMRT and N-CoR may utilize similar but distinct mechanisms for interaction with nuclear receptors.

We cannot exclude the possibility that the tight association with SMRT by the AF2-AD deletion mutants may weaken hormone binding to the receptor, but the ability of RAR403 to respond to ligand treatment in yeast cells indicates that this mutation does not eliminate the receptor's hormone-binding capability, consistent with previous observations (14, 53). Therefore, the inability of hormone to dissociate corepressors is likely due to the lack of certain conformational changes that would normally take place in the presence of the AF2-AD. It is possible that the assumed shift of AF2-AD upon hormone binding is a prerequisite for additional structural changes that are important for corepressor dissociation. Alternatively, the shift of helix 12 may mask or compete with certain interacting surfaces required for binding corepressors. The fact that the AF2-AD deletion creates a mutant that binds tighter to the corepressors favors this model. We suspect that helix 11 could constitute such an interacting surface, since disruption of this helix eliminates the enhanced interaction resulting from deletion of AF2-AD. Our results suggest that AF2-AD may act to balance the association between nuclear receptors and the corepressors, by preventing over-association of unliganded receptors with corepressors, thereby facilitating ligand-dependent dissociation of corepressors.

Nested deletion analysis reveals two distinct subdomains in SMRT that are capable of independent interaction with nuclear receptors. These two recep-

tor-interacting domains, RID-1 and RID-2, interact differently with TR and RAR. The N-terminal RID-1 region interacts more strongly with RAR, and it contains a glutamine-rich domain, while the C-terminal RID-2 region interacts better with TR and contains a putative helical domain analogous to that identified previously in N-CoR (25). The different receptor-interacting properties of these two domains suggest that SMRT may utilize distinct mechanisms for interaction with different receptors. The RID-2 region in N-CoR has been shown to interact directly with the hinge region of TR (25), and therefore it is reasonable to predict that the N-terminal RID-1 region might interact with the C-terminal region of the LBD.

Functional analysis of the transcriptional repression activities of SMRT and N-CoR reveals two independent domains that are capable of repressing basal transcription. Together, there appear to be four independent repression domains in N-CoR and two in SMRT. These repression domains could act independently, and some repress basal transcription as efficiently as the full-length protein, suggesting that these domains might act redundantly and possibly through different mechanisms. Sequence comparison of these repression domains gives little clue as to possible mechanisms of repression. However, within SRD-1 and the corresponding NRD-3, four potential repeated motifs sharing a consensus sequence of GSITQGTPA have been identified (32). In addition, two other potential repeats with a consensus sequence of KGHV●YEG are noted. These motifs are well conserved between SMRT and N-CoR, suggesting that they might contribute to repression.

Recently, several papers reported that mSin3A and the histone deacetylase HDAC1 form a ternary complex with SMRT and N-CoR (42, 46). These results indicate that SMRT and N-CoR, while interacting with unliganded receptors, can also interact with additional corepressors such as mSin3A and mSin3B (54), as well as the histone deacetylases HDAC1 (55) and mRPD3 (56). The recruitment of histone deacetylase to target promoters by unliganded receptors through SMRT, N-CoR, and mSin3 suggests that deacetylation of histones or other factors may play a role in transcriptional repression, perhaps by establishing an unfavorable chromatin structure for transcriptional activation (41). Our results suggest weak two-hybrid interactions between SMRT/N-CoR and mSin3A, or between mSin3A and HDAC1, even though a VP16 activation domain was present. Alternatively, these results may suggest that the repression activity of the corepressor complex is dominant over that of the VP16 activation domain. An *in vitro* protein-protein interaction assay detects association of mSin3A with NRD-1 and NRD-4, but not with other repression domains. Although our results are consistent with recent reports, our data also suggest the possibility of other repression mechanisms.

MATERIALS AND METHODS

Plasmids

The GST fusions of C-SMRT (GST-SMRT) and RXR (GST-RXR) were described previously (24, 30). Serial C-terminal and N-terminal deletion mutants of human TR β and human RAR α were generated by appropriate restriction enzyme digestion and/or PCR amplification from the parental expression construct pCMX-hTR β and pCMX-hRAR α (57). The GST-SMRT deletion constructs were generated by enzyme digestion at indicated residues from the parental construct GST-SMRT. The Gal4 DBD fusions of individual repression domains of SMRT and N-CoR were generated by PCR amplification and were subsequently transferred to pGEX vector for expression of GST fusion proteins. The VP16 AD-mSin3A construct was created by subcloning the Scal (at residue 56) to BglII fragment of mSin3A (58) into the pCMX-VP16 vector. Detailed information regarding these plasmids is available upon request.

Far-Western Analysis

GST fusion proteins were separated by denaturing protein gels (SDS-PAGE) and electroblotted onto nitrocellulose filters in transfer buffer (25 mM Tris-HCl, pH 8.3; 192 mM glycine; 0.01% SDS). After denaturation in 6 M guanidine hydrochloride (GnHCl), the proteins were renatured by stepwise dilution of GnHCl to 0.187 M in HB buffer (25 mM HEPES, pH 7.7; 25 mM NaCl; 5 mM MgCl₂; 1 mM dithiothreitol). The filters were then saturated in blocking buffer (5% nonfat milk, then 1% milk in HB buffer plus 0.05% NP40) at 4°C overnight or at 37°C for 1 h. *In vitro* translated ³⁵S-labeled proteins were diluted into hybridization buffer (20 mM HEPES, pH 7.7; 75 mM KCl; 0.1 mM EDTA; 2.5 mM MgCl₂; 0.05% NP40; 1% milk; 1 mM dithiothreitol), and the filters were allowed to hybridize overnight at 4°C. After three washes (5 min each) with the hybridization buffer, the bound proteins were detected by autoradiography.

Yeast Two-Hybrid Assay

The yeast two-hybrid assay was carried out in the Y190 yeast strain (59). The Gal4 DBD fusion constructs were generated in either the pAS or pGBT vector (CLONTECH, Palo Alto, CA), and the Gal4 AD fusion constructs were in the pGAD or pACT vector (CLONTECH). The β -galactosidase activities were determined with the O-nitrophenyl β -D-galactopyranoside (Sigma, St. Louis, MO) liquid assay as previously described (30).

Cell Culture and Transient Transfection

African green monkey kidney CV-1 cells were grown in DMEM supplemented with 10% resin-charcoal stripped FBS, 50 U/ml penicillin G, and 50 μ g/ml streptomycin sulfate at 37°C in 5% CO₂. One day before transfection, cells were plated in a 24-well culture dish at a density of 50,000 cells per well. Transfection was performed by standard calcium phosphate precipitation (57). All transfection experiments were performed in triplicate and were replicated at least once. Twelve hours after transfection, cells were washed with PBS and refed fresh medium containing indicated amounts of ligands. After 30 h, cells were harvested for β -galactosidase and luciferase assay as described previously (30). The relative luciferase activities are arbitrary light units normalized to the β -galactosidase activities.

In Vitro Translation and Western Blot

In vitro transcription/translation reactions were carried out in rabbit reticulocyte lysates using the TNT T7 Quick coupled

transcription/translation system (Promega, Madison, WI). [³⁵S]Methionine (Amersham, Arlington Heights, IL) was added during the translation reactions, which were performed at 30°C for 90 min. The translated reactions were analyzed by SDS-PAGE, followed by autoradiography. For Western blot analysis, transfected cells were lysed in SDS-sample buffer, and the extracts were separated by SDS-PAGE. The gels were transferred onto nitrocellulose membranes, blocked with nonfat milk, and hybridized with anti-Gal4 DBD monoclonal antibody according to manufacturer's recommendation (Santa Cruz Biotechnology, Santa Cruz, CA). The filters were washed and incubated with horseradish peroxidase-conjugated anti-mouse IgG secondary antibody and developed by enhanced chemiluminescent reaction (Amersham).

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Coactivation and Corepression in Transcriptional Regulation by Steroid/Nuclear Hormone Receptors

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ABSTRACT: Transcriptional regulation by steroid/nuclear receptors is the central theme of hormone action that controls key aspects of cell differentiation, development, and homeostasis. The molecular mechanisms of gene activation and repression by the receptors have been investigated extensively in recent years. Particularly, several new proteins involved in this signaling pathway have been identified, cloned, and demonstrated to modulate transcription in concert with nuclear receptors. In the absence of hormone, unliganded receptors interact with a family of transcriptional corepressors, including SMRT and N-CoR, which target histone deacetylases to establish a condensed and repressed chromatin structure. Upon hormone binding, the corepressor complex is replaced by a coactivator complex, containing SRC1/TIF2/RAC3 and CBP/p300, which target histone acetyltransferases to generate a transcriptionally accessible chromatin structure. These studies initiate a new era in the history of hormone research and provide novel entry points for understanding the mechanisms of transcriptional regulation by steroid/nuclear receptors.

KEY WORDS: coactivators/corepressors, SMRT/N-CoR, histone acetylation deacetylation, RAC3/SRC-1/TIF2, CBP/p300.

I. INTRODUCTION

Transcriptional regulation by steroids, thyroids, retinoids, and vitamin D₃ plays a critical role in controlling numerous key aspects in animal development, reproduction, homeostasis, metabolism, and adult organ physiology.^{7,57,71,72,102} The intracellular receptors for these hormones and lipophilic compounds comprise a large family, many of which are ligand-dependent transcription factors. These receptor proteins are characterized by a common domain structure: an N-terminal DNA-binding domain (DBD) plus a C-terminal ligand binding domain (LBD). In addition to ligand binding, the LBD also mediates dimerization, transcriptional activation, and repression. Two classes of the receptors are known to mediate the function of all identified hormones. The first class contains receptors for steroids such as progesterone (PR), glucocorticoid (GR), estrogen (ER), androgen (AR), and minera-

corticoid (MR). These steroid receptors are normally inactive and associated with heat shock proteins (HSPs) in the absence of hormone. Hormone binding activates the receptors by dissociating HSPs, facilitating homodimerization, nuclear translocation, and eventually allows the receptors to bind DNA and control gene expression. The second class includes receptors for vitamin D₃ (VDR), thyroids (TR), retinoids (RAR),³⁶ rexinoid (RXR),⁷⁶ and prostanoids (PPAR).^{34,59} This class of receptors are nuclear proteins that form heterodimeric complexes with RXR.⁷¹ Several of these RXR heterodimers are thought to bind DNA and repress transcription in the absence of ligand and activate transcription upon ligand treatment.^{35,60,107,114,126}

In the past few years, the mechanisms of transcriptional activation and repression by steroid/nuclear receptors have been the focus of intense studies. In particular, new regulatory proteins that bind to the receptors have been explored exten-

sively (see Table 1 and Refs. 37,51,94), by utilizing biochemical and genetic screening strategies.^{20,28,30} These studies have led to the discovery of many putative transcriptional coactivators and corepressors that associate with either liganded or unliganded receptors, respectively. Recent studies on mechanisms of coactivation and corepression by these receptor cofactors have established a molecular link between transcriptional activation and enzymatic modification of chromatin. In this review, we provide a survey of current putative nuclear receptor coactivators and corepressors and suggest mechanisms of coactivation and corepression. In particular, for coactivators, we focus the discussion on a novel family of proteins that includes steroid receptor coactivator-1 (SRC-1),^{56,81,100,122} transcriptional intermediate factor-2 (TIF2),¹⁰⁹ glucocorticoid receptor interacting protein-1 (GRIP1),⁴⁷ and receptor-associated coactivator 3 (RAC3),⁶⁸ p300/CBP/cointegrator protein (pCIP),¹⁰⁵ activator of retinoid receptors (ACTR),¹⁶ and amplification in breast cancers (AIB1).² For corepressors, we discuss the mechanisms of corepression by two related proteins: the silencing mediator for retinoid and thyroid hormone action (SMRT)¹⁷ and nuclear receptor corepressor (N-CoR).⁵⁰

II. RECEPTOR-ASSOCIATED COACTIVATORS

The involvement of coregulatory proteins in receptor signaling was first postulated when members of nuclear receptor superfamily were found to functionally cross-react with each other⁷⁴ and with other classes of transcription factors.⁹⁰ Since then, biochemical and genetic approaches have been used successfully in identifying and cloning receptor-associated proteins.^{11,91} In one approach, purified glutathione-S-transferase (GST)-receptor fusion proteins are incubated with metabolically labeled cell extracts prepared before or after hormone treatments. The cellular proteins bound to GST fusion proteins are collected and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). Using this approach, two major ER-associated proteins (ERAP140 and ERAP160) and a 300-kDa protein were identi-

fied.^{11,39} Similarly, two proteins with molecular weight 140 kDa (p140) and 160 kDa (p160) were found to interact with liganded RAR and RXR,⁶¹ and three proteins with molecular weight 95, 120, and 170 kDa were found to associate with liganded GR.²⁹ In an alternative approach, stably transfected cells expressing epitope-tagged TR were used in immunoprecipitations to isolate TR-associated proteins (TRAPs).³² Nine polypeptides in the TRAP complex were identified: TRAP80, 93, 95, 97, 100, 150, 170, 220, and 230. The TRAP complex appears to enhance TR-mediated transcriptional activation *in vitro*, suggesting that at least certain components act as coactivators. Whether any of these polypeptides interacts directly with liganded TR, and whether all of these TRAPs are required for T3 stimulated transcription, await further investigation. Using other approaches, the yeast two-hybrid system^{28,30,41} and expression screening of bacteriophage cDNA libraries,¹¹ investigators have cloned most of the current candidates for receptor-associated cofactors.

The receptor-associated coactivators (RACs) are proteins that physically interact with DNA-bound and transcriptionally active nuclear receptors, enhancing transcriptional activation by the receptors. Several proteins have been demonstrated to meet these criteria, including the transcriptional intermediate factor 1 (TIF1),⁶⁶ the receptor interacting protein 140 (RIP140),¹² the androgen receptor activator (ARA70),¹²³ and the SRC gene family.^{2,16,56,68,81,100,105,122,130} In addition, the human SWI/SNF complex^{19,58,75} and the CREB/E1A-binding protein (CBP/p300)^{3,21,62} can also interact with nuclear receptors and enhance transcription, despite their broader roles in transcriptional activation associated with other transcription factors. These "general" coactivators are thought to function as integrators for different signaling pathways. Among these putative RACs, members of the SRC family (Figure 1) have been the major focus of recent studies. Their roles in transcriptional activation of the receptors have been strongly supported, and recent observations suggest that some SRC proteins may contribute to the development of human cancers.² Below, we discuss the cloning, characterization, and mechanisms of coactivation by the SRC family proteins.

TABLE 1
Putative Nuclear Receptor Coactivators and Corepressors

| | Species | Synonyms | Homologs | Related proteins | Receptors | Functional properties | Refs. |
|---------------------|---------|------------------|-----------------|------------------|-----------------------------------|--|---------------|
| Coactivators | | | | | | | |
| RIP140 | Human | ERAP140 p140? | | | ER | Stimulate or repress ER function, depending on level of expression | 12 |
| TRIP1 | Human | Sug1 | mSug1, ySug1 | | TR | A component of the 26S proteasome | 67 |
| TIF1 | Human | TIF1 α | TIF1 β | KRIP-1, T18 | ER, RAR, RXR | Stimulate or repress transactivation by receptor depending on level of expression | 26,65 |
| SRC-1 | Human | NCoA-1 | mSRC-1 | TIF2, RAC3 | | Potentiate receptor activation | 56,81, 122 |
| TIF2 | Human | | GRIP1 | SRC-1, RAC3 | | Potentiate receptor activation | 109 |
| GRIP1 | Mouse | NCoA-2 | TIF2 | SRC-1, RAC3 | | Potentiate receptor activation in yeast | 47 |
| ARA70 | Human | | | | AR, GR, ER, PR | Potentiate AR activation | 123 |
| CBP | Mouse | | | p300 | RAR, RXR, TR | Potentiate transactivation by receptors and other transcription factors | 13,56 |
| p300 | Human | | | CBP | RAR, RXR, TR | Potentiate transactivation by receptors and other transcription factors | 13,56 |
| SWI2 | Human | SNF2 | hBRG1 | | RAR, ER | Enhance receptor activation | 19 |
| p/CIP | Mouse | | RAC3 | TIF2, SRC-1 | ER, TR, RXR, RAR, | Potentiate activation by receptors and STATs | 105 |
| RAC3 | Human | ACTR, AIB1 | p/CIP | SRC-1, TIF2 | RAR, RXR, TR, VDR, ER, PR | Potentiate activation by nuclear receptors | 68 |
| Corepressors | | | | | | | |
| SMRT | Human | TRAC-2 | mSMRT | N-CoR | TR, RAR, COUP, RVR, Rev-erb | Mediate transcriptional silencing by unliganded receptors | 17 |
| N-CoR | Mouse | RIP13 | hN-CoR | SMRT | TR, RAR, COUP, RVR, Rev-erb | Mediate silencing by unliganded receptors | 50 |
| mSin3A | Mouse | | | mSin3B | | Associate with SMRT and N-CoR | 44,79 |
| mSin3B | Mouse | | | mSin3A | | Associate with N-CoR | 44,79 |
| HDAC1 | Human | HD1 | | RPD3 | | Associate with SMRT/ N-CoR and mSin3 complex, histone deacetylase | 42 |
| mRPD3 | Mouse | | yRPD3 | HDAC1 | | Associate with SMRT/ N-CoR and mSin3 complex, histone deacetylase | 44 |

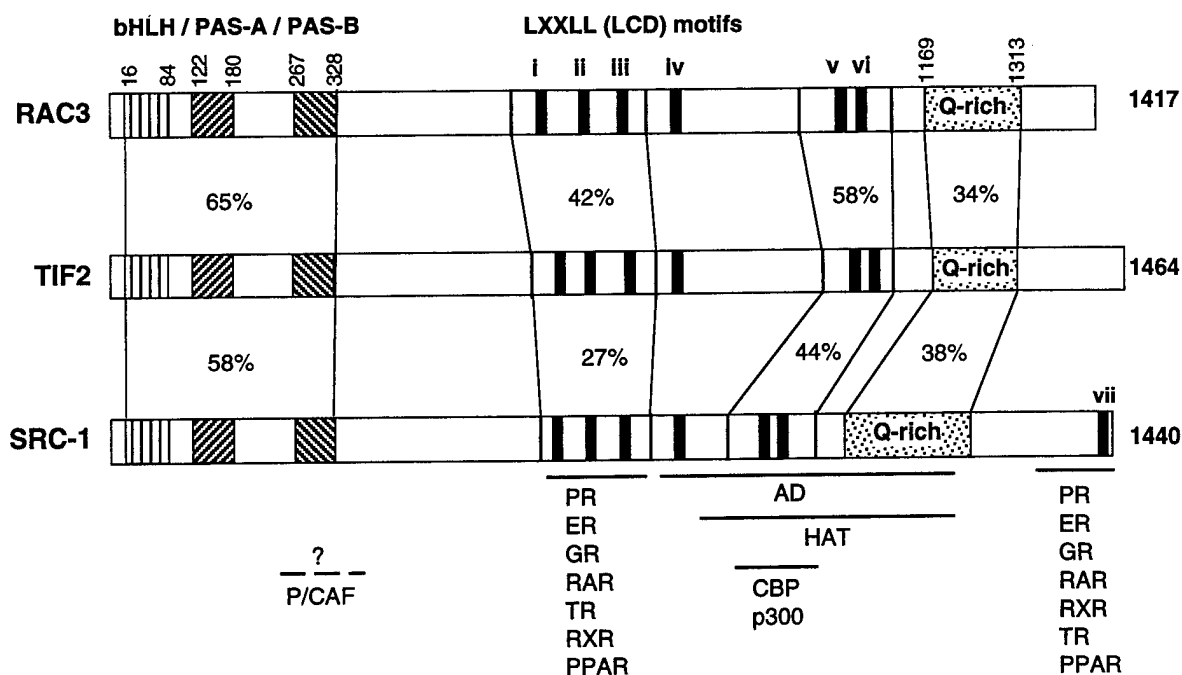


FIGURE 1. The SRC family of receptor-associated coactivators. The human proteins in this gene family are used for comparison. RAC3 is also known as ACTR and AIB1, with a few amino acid differences. p/CIP is the mouse homologue of RAC3. TIF2 is also known as GRIP1 or NCoA-2. The similarity of each domain between RAC3 and TIF2, and between RAC3 and SRC-1 are as indicated. The N-terminal region of these three proteins contains putative basic-helix-loop-helix (bHLH), Per-AhR-Sim (PAS)-A and B domains. The central region of these proteins contains six LXXLL (or LCD: leucine charged domain) motifs. The C terminal contains a glutamine-rich domain where a consecutive 26 glutamine residues was found in RAC3 but not in TIF2 or SRC-1. A central receptor interacting domain is located around the first three LXXLL motifs, while a second interacting domain is found at the C-terminus of SRC-1. The nuclear receptors that have been identified to interact with these regions are shown. A single activation domain (AD) and a histone acetyltransferase domain (HAT) as well as a region involved in CBP/p300 interaction are also indicated.

A. SRC-1 (NCoA-1)

SRC-1 was initially identified as a 1061-aa polypeptide that interacts with PR.⁸¹ Subsequently, additional 1278 base pairs (bp) at 5' of SRC-1 were reported.¹⁰⁰ This N-terminal sequence predicts an additional 362 amino acids. Therefore, the human SRC-1 gene appears to encode a 1440-aa polypeptide with an estimated molecular weight of 156 kDa, which is consistent with the putative coactivator p160 by previous biochemical studies.⁵⁶ Three laboratories also reported the mouse homologue of SRC-1, which appears to encode additional N-terminal sequences^{56,122,130} and shares more than 90% identity with human SRC-1. Northern blot analysis indicates two SRC-1 transcripts in most human

tissues and cell lines, consistent with the cloning of several spliced variants of mSRC-1.⁵⁶

Full-length SRC-1 contains a putative N-terminal basis-helix-loop-helix (bHLH) domain common to many transcriptional regulators.¹²² This bHLH domain functions as a DNA-binding motif as well as a dimerization interface for many transcriptional factors, including the MyoD family of proteins.^{77,78} However, the function of the bHLH motif in SRC-1 remains unclear. Downstream from the bHLH motif, a region similar to the Period-Aryl hydrocarbon receptor-Single minded (PAS)-A and B domains was identified. The PAS domains in *Drosophila*, as well as in hypoxia and dioxin signaling pathways, have been demonstrated to play important roles in protein-protein interaction, heterodimeric partner selection, and

target gene specificity.^{70,98,129} Nonetheless, both bHLH and PAS domains seem to be dispensible for SRC-1 coactivation.⁸¹ Following the PAS domain is a region rich in serine and proline residues and a glutamine-rich domain. Despite the identification of these special features in the SRC-1 sequence, no functional role has been attributed to any of these domains.

SRC-1 interacts directly with agonist (R5020) but not antagonist (RU486)-bound PR in a GST pull-down assay,⁸¹ as well as with RAR, ER, TR, PPAR, VDR, and RXR in either GST pull-down, far-Western blot, or two-hybrid assays.^{25,56,81,100,122,130} In addition, Gal4 DBD-SRC-1 fusion stimulates transcriptional activation from a Gal4-dependent promoter,^{68,105,130} indicating that SRC-1 possesses intrinsic activation function. The capability of a protein to associate with nuclear receptors and to activate transcription supports the role of SRC-1 as an auxillary factor in receptor-mediated transcription. Two domains in SRC-1 interact independently with the receptors, the first beyond the glutamine-rich region at the C-terminus⁸¹ and a second at the central region between amino acids 569 and 789.^{122,130} Further analysis of these interacting regions revealed striking conservation of motifs with a LXXLL consensus core sequence (or leucine charged domains; LCDs) flanked by charged residues.^{43,68,105} Three such motifs (i, ii, iii) were found in the second interacting domains and one motif (vii) was identified within the C-terminus interacting domain. Each of these four motifs is sufficient to interact with liganded ER, and point mutations in all four motifs disrupt ER-receptor interaction.⁴³ Furthermore, a synthetic polypeptide encompassing the C-terminus motif inhibits interaction between wild-type SRC-1 and ER. Deletion analysis and peptide competition assay demonstrated that motif ii was most important in mediating the interaction with both ER and RAR, whereas motif i was less critical and motif iii is not required.¹⁰⁵ Somewhat paradoxically, motif iii mutation alone seemed to abolish the ability of SRC-1 to rescue RAR-dependent transcription blocked by specific IgG, but had no effect on ER-dependent transcription. On the other hand, motif ii mutation seemed to exert a dramatic effect on ER-dependent transcription, but a less obvious effect on RAR-dependent transcrip-

tion. These studies suggest that the interacting surfaces between SRC-1 and the receptors are multifaced, indicating that the helical interaction motifs may dictate receptor specificity. Similar LXXLL motifs were found in other receptor-associated proteins such as RIP140, TIF1, CBP/p300, and several TR-interacting proteins,⁹¹ suggesting the this motif may be a common feature involved in receptor interaction. Intriguingly, introduction of motif iv polypeptide was able to selectively inhibit RAR-dependent, but not STAT-dependent transcriptional activation¹⁰⁵ and a synthetic oligopeptide corresponding to the STAT-interacting domain of CBP selectively blocked STAT-dependent activation and had no effect on RAR-dependent activation. These data further suggest that these oligopeptides could be useful as selective agents for blocking specific signaling pathways. It needs to be noted that not all LXXLL consensus sequences mediate interaction with nuclear receptors. For example, within the activation domain of SRC family proteins, at least three additional LXXLL motifs were identified that appear to not be involved in protein-protein interaction with liganded receptors.

Functional studies have demonstrated that SRC-1 enhances transcriptional activation by several different receptors.^{45,53,73,96,130} Transient transfection of the truncated SRC-1 lacking the N-terminal bHLH-PAS region enhances progesterone (R5020)-stimulated PR-dependent transcription without altering basal promoter activity, but has no effect on activity of RU-486 antagonist-bound PR.⁸¹ This result suggests that the bHLH-PAS domain is dispensible for coactivation. SRC-1 also enhances ER, GR, TR, PPAR, and RXR transcriptional activities, but has no effect on E2F- or forskolin-stimulated transcription.^{25,81,130} Importantly, SRC-1 can reverse the cross-inhibitory effect of E2 on R5020-stimulated transcription, and C-terminus receptor-interacting domain alone can inhibit hormone-stimulated PR and TR transcriptional events.⁸¹ These data suggest that SRC-1 is perhaps a limiting coactivator shared by different nuclear receptors. Microinjection of anti-SRC-1 IgG inhibits transcription from RARE, ERE, TRE, and PRE-driven *lacZ* reporters and had no effect on Sp1 or CMV-driven reporters,¹⁰⁵ consistent with the results obtained with

transient transfections.⁸¹ Furthermore, coinjection of a wild-type SRC-1 expression vector could restore RA-dependent transcription inhibited by anti-SRC-1. These data demonstrate that SRC-1 is required for receptor-mediated transcriptional activation.

B. GRIP1/TIF2

After the report of SRC-1, a 812-aa protein fragment known as glucocorticoid receptor interacting protein 1 (GRIP1) was cloned from a mouse cDNA library in a yeast two-hybrid screen using GR LBD as bait.⁴⁸ Full-length GRIP1 cDNA was subsequently isolated and an open reading frame of 1462-aa with an estimated molecular mass of 158.5 kDa was predicted.⁴⁸ Sequence comparison between full-length GRIP1 and SRC-1 revealed that these two proteins are highly related and share approximately 40% identity (Figure 1). The similarity is especially striking at the N-terminal bHLH-PAS domain (58% identity), suggesting that GRIP1 and SRC-1 belong to the same gene family. GRIP1 was shown to interact with all five steroid receptors (GR, ER, AR, MR, and PR) in a hormone and AF2-dependent manner, and the Gal4 DBD-GRIP1 fusion was shown to stimulate transcription both in yeast and in mammalian cells.^{47,112} The ability of GRIP1 to interact with liganded receptors and to stimulate transcription supports its role in receptor coactivation. Indeed, expression of GRIP1 enhances ligand-dependent transcriptional activation by all steroid receptors, as well as several class II nuclear receptors, including VDR, RAR, and TR in yeast.^{47,112} On the other hand, transient transfection experiments in mammalian cells suggested that the 812-aa GRIP1 fragment inhibited rather than activated transcription from the MMTV and CMV promoter in mouse L cells.⁴⁸ The reason for this inhibitory effect is unclear.

TIF2 was isolated in search of the 160-kDa protein(s) that interacts with liganded GST-ER and GST-RAR fusion proteins in biochemical assays.¹⁰⁹ By screening a human bacteriophage cDNA library with ³²P-GST-ER, TIF2 was identified and shown to encode a 1461-aa protein with a predicted molecular weight of 159 kDa.

TIF2 is highly related to GRIP1, sharing over 94% identity, indicating that GRIP1 and TIF2 are the mouse and human orthologs. TIF2, like SRC-1, was demonstrated by immunodepletion studies to be a major component of the biochemically characterized p160 proteins.^{11,39,61} As with GRIP1, TIF2 interacts with several liganded receptors, including ER, RAR, RXR, and TR. Furthermore, point mutation within the AF2-AD core abolish the binding, supporting the idea that GRIP1/TIF2 is a common and AF2-dependent transcriptional coactivator for nuclear receptors. It was shown that transiently transfected full-length TIF2 accumulates in specific nuclear domains. Conversely, a central fragment of TIF2 (TIF2.1), containing only the receptor-interacting and transcriptional activation domains, remains dispersed in the cytoplasm. Cotransfection of TIF2.1 with RAR, ER, or PR induces an agonist-dependent translocation of the TIF2.1 fragment from the cytoplasm into the nucleus where they remain dispersed. These observations demonstrate an *in vivo*, physical interaction between TIF2 and the liganded receptors. It is not clear whether the discrete nuclear localization of TIF2 is a natural phenomenon or an artificial condition due to overexpression. Related to this, both transfected and endogenous SRC-1 protein localizes uniformly in the nucleus, but colocalizes with p300 at specific nuclear domains after cotransfection. In addition, TIF2 also contains a strong autonomous transcriptional activation function, and overexpression of TIF2 appears to relieve, at least partially, the squelching effect generated by overexpression of an increasing amount of ER.¹⁰⁹ Furthermore, overexpression of TIF2 enhances transcriptional activation by ER, AR, and PR in an agonist-specific manner, confirming the function of TIF2 as a transcriptional coactivator.

Recently, a mouse splicing variant of GRIP1 was isolated and named NCoA-2.¹⁰⁵ NCoA-2 appears almost identical to GRIP1, except for two obvious unrelated gaps. Therefore, TIF2, GRIP1, and NCoA-2 are the products of a single gene. It was demonstrated in immunoinjection experiments that anti-NCoA-2 IgG could not block RAR-dependent transcriptional activation. However, coinjection of a NCoA-2 expression vector reverses the inhibition of RAR-dependent transcrip-

tion blocked by injection of anti-NCoA-1 (SRC-1) IgG.¹⁰⁵ These studies suggest that both SRC-1 (NCoA-1) and TIF2 (NCoA-2) are sufficient for mediating RAR-transactivation. Although these studies suggest that NCoA-2 is less critical in receptor activation, in transient transfections TIF2 markedly enhances transcriptional activation by nuclear receptors compared to SRC-1 and RAC3.

C. RAC3/pCIP/ACTR/AIB1

A third member of the SRC family has been recently identified and cloned in several laboratories.^{2,16,68,105} This protein is known as receptor-associated coactivator 3 (RAC3),⁶⁸ p300/CBP/cointegrator protein (p/CIP),¹⁰⁵ activator of retinoid receptors (ACTR),¹⁶ and amplification in breast cancer 1 (AIB1).² Like SRC-1 and TIF2, RAC3 interacts with a number of nuclear receptors in an AF-2 and ligand-dependent manner and RAC3 possesses intrinsic transcriptional activation function in both yeast and mammalian cells.⁶⁸ In transient transfections, RAC3 potentiates ligand-dependent transcriptional activation of both RAR and PR in mammalian cells. Comparison of the central domains of SRC1, TIF2, and RAC3 revealed seven highly conserved motifs with a core consensus sequence of LXXLL flanked by highly charged residues (Figure 1). The three N-terminal motifs have been demonstrated to function as critical regions for mediating protein-protein interaction with liganded receptors.^{43,105} The other three motifs are located within the transcriptional activation CBP/p300 interacting domains.^{68,105,122} Sequence comparison among RAC3, TIF2, and SRC-1 reveals that these three genes are highly related to each other and the conservation is especially striking at the N-terminal bHLH-PAS region. It also appears that RAC3 is more related to TIF2 (65% similarity) than to SRC-1 (59% similarity). Intriguingly, RAC3 contains a stretch of about 26 consecutive glutamine residues located within the glutamine-rich domain. No such consecutive glutamine residues were found in either SRC-1 or TIF2. It is worth noting that a similar motif is also present in several transcription factors, including the AR, where the length of the poly-Q domain has been implicated in the development of prostate can-

cers.¹⁴ The function of this domain in RAC3 is currently unclear.

p/CIP was identified by expression screening of a bacteriophage mouse cDNA library using ³²P-labeled GST-CBP protein as a probe.¹⁰⁵ Immunoprecipitation of HeLa cell extract using anti-p/CIP antibody pulled down more CBP/p300 than using antibodies against SRC-1 or TIF2,¹⁰⁵ suggesting that the vast majority of CBP/p300 are associated with p/CIP. Therefore, p/CIP was purported to function as a subunit in the cointegrator complex containing CBP/p300 and its associated factor p/CAF.¹²¹ However, RAC3/pCIP is expressed at higher levels in HeLa cells compared to TIF2 and SRC-1 and *in vitro* interaction assays demonstrate that both RAC3 and SRC-1 associate well with CBP.^{69a} Thus, the proposed relative contribution of these three coactivators in the integrator complex is still unclear. Furthermore, microinjection of anti-p/CIP IgG directly demonstrates that blockage of p/CIP function selectively inhibits transcriptional activation by RAR, ER, TR, and PR, but not by SP-1 or from the cytomegalovirus (CMV) promoter. This inhibitory effect could be rescued by coinjection of p/CIP and CBP expression vectors, suggesting that CBP/pCIP are required together for nuclear receptor activation. A core CBP-interacting domain of p/CIP completely inhibits RA-dependent gene activation. Immunoinjection of anti-p/CIP IgG also abolished STAT-dependent and TPA-dependent transcriptional activation, suggesting that p/CIP is required for transcriptional activation by other CBP-dependent transcription factors. On the other hand, anti-NCoA-1/SRC1 IgG does not block cAMP- or IFN γ -dependent reporters actively, whereas immunoinjection of this antibody efficiently inhibits transcriptional activation from several receptors. This repression is reversed by injecting expression vectors for either NCoA-1/SRC-1 or NCoA-2/TIF2, but not for p/CIP. Together, these studies imply that NCoA-1/SRC1 is selectively required as a coactivator for nuclear receptors, whereas the CBP/pCIP complex plays a more general role in gene activation.

ACTR was isolated in a yeast one-hybrid screen for hRAR β -stimulatory proteins¹⁶ and was shown to enhance ligand-dependent transcriptional activation by hRAR β in yeast. The cofactor is ex-

pressed in a tissue and cell type-specific manner, with high levels of expression in heart, skeletal muscle, pancreas, and placenta as well as in certain cell lines. In transient transfections, overexpression of ACTR enhances ligand-dependent transcriptional activation of RAR, TR, RXR, and GR about two- to threefold, similar to that observed with RAC3 and p/CIP.^{68,105} The reason for the low level of enhancement in transient transfection by ACTR, p/CIP, and RAC3 compared to SRC-1⁸¹ is unclear. Perhaps the stoichiometry between the receptor and individual coactivators may be critical in controlling the actual level of enhancement. Similar to RAC3 and p/CIP, ACTR interacts with members of nuclear receptors in a ligand- and AF2-dependent manner. In addition, ACTR contains two independent receptor-interacting domains, and associates with liganded receptors on DNA elements. The two interacting domains cover the regions containing the LXXLL motifs, consistent with the recent finding that these motifs are critical and perhaps sufficient for mediating protein-protein interaction with nuclear receptors.^{43,105} The transcriptional activation domain of ACTR was mapped to between amino acid 1018 and 1290, consistent with the observation in RAC3^{69a} and p/CIP.¹⁰⁵ Like SRC-1, this activation domain interacts directly with CBP/p300, suggesting that one mechanism of activation by RAC3/ACTR/pCIP is to recruit CBP/p300. Furthermore, ACTR interacts with P/CAF,¹¹³ and both function as histone acetyltransferases. Interestingly, ACTR, RAC3, and SRC-1 each possess intrinsic histone acetyltransferase activity that maps to a region overlapping with the CBP/p300-interacting and transcriptional activation domains.¹⁶ These studies further strengthen the hypothesis that histone acetylation is one of the mechanisms of transcription stimulation by nuclear receptor coactivator complexes.¹¹⁰

AIB1 was isolated during a search on the long arm of chromosome 20 for genes whose expression and copy numbers alter in human breast cancers.² AIB1 is amplified and overexpressed in four out of five ER-positive breast and ovarian cancer cell lines. This gene is also found amplified in approximately 10% of the primary breast tumors and is overexpressed in a majority of the primary breast tumors analyzed. AIB1 protein

interacts with ER in a ligand-dependent fashion, and transfection of AIB1 enhances E2-dependent transcription. These observations suggest that altered expression of AIB1 may contribute to the development of steroid-dependent cancers. Similarly, both RAC3 and ACTR are overexpressed in several human cancer cell lines, including Burkitt's lymphoma Raji cells and colorectal adenocarcinoma SW480 cells.¹⁶ In Burkitt's lymphoma cells, both RAC3 and TIF2, but not SRC-1, appear to be highly expressed, whereas in colorectal adenocarcinoma, all three coactivators are overexpressed. The functional significance of this altered expression remains to be investigated.

RAC3, ACTR, and AIB1 appear to be encoded by a single human gene, with only subtle amino acid changes, whereas p/CIP is likely a mouse homologue of the same gene. Comparison of p/CIP and RAC3 sequences indicates they share over 76% identity in aa sequence with three major differences: (1) an unrelated gap between amino acids 172 and 197 in the N-terminal bHLH-PAS region, (2) a change in the relative position of the poly-Q region, and (3) another unrelated sequence at the C-terminus 103 amino acids. However, the nucleotide sequences of these two clones share over 80% identity, including the two unrelated gaps. A more detailed comparison between p/CIP and RAC3 suggests that these two genes are not splicing variants, but that the major differences are due to reading frame change. We have reconfirmed the RAC3 sequence and because all three human genes have almost identical sequences, it is possible that the changes in the reading frame of p/CIP might be due to a sequencing error or that the human gene has evolved away from the mouse gene in these places. The implication of the change in the relative position of the poly-Q domain between the human and mouse genes is unclear. It is noted that this domain seems to be located at either side of an important functional domain involved in transcriptional activation, CBP/p300 interactions, and histone acetyltransferase activity. Therefore, this poly-Q domain may have a role in regulating the functional specificity of this domain.

Overall, these studies suggest that malfunction of the SRC proteins may contribute to the pathogenesis of human cancers, especially for

those regulated by hormones. The cloning of these novel SRC family proteins, the discoveries of their enzymatic activities, and the elucidation of their binding partners have helped to establish a more complete signaling pathway from liganded receptors to chromatin structure and gene activation, regulated primarily by direct protein-protein interactions. It is still unclear how these three coactivators work. It is also unclear whether they modify the same substrate. Recent studies using microinjected, single-stranded DNA in *Xenopus* oocytes suggest that nucleosome disruption is insufficient for gene activation by TR,¹¹⁸ indicating a requirement of other components in receptor-mediated gene activation. Coordinately, the relationship between the coactivators and basal transcriptional machinery is still elusive, and the role of individual coactivators in different receptor signaling events remains unanswered. Further investigation will provide more insights into the nature of transcriptional activation by nuclear receptors and the mechanisms of coactivation.

III. RECEPTOR-ASSOCIATED COREPRESSORS

In addition to transcriptional activation, several nuclear receptors can also repress basal transcription in the absence of ligand.^{22,40,119,124} Importantly, repression by TR, RAR, and their mutants plays a critical role in controlling oncogenesis and cellular differentiation.^{23,38,86,106} Overexpression of TR and RAR LBDs block transcriptional repression by the wild-type receptors both *in vivo*^{5,10} and *in vitro*,¹⁰⁴ presumably through competing for a limiting corepressor(s). Biochemical studies revealed at least two polypeptides of 270 and 170 kDa that appear to associate with unliganded TR and RAR.⁵⁰ By using the yeast two-hybrid screening system, a 168-kDa protein termed silencing mediator for RAR and TR (SMRT) and a 270-kDa protein named nuclear receptor corepressor (N-CoR) were identified and cloned.^{17,50} SMRT and N-CoR meet the criteria for receptor-associated corepressors, including physical interaction with transcriptionally repressive receptors and enhancement of transcriptional repression by the receptors.

SMRT was originally identified and cloned from a human B-cell cDNA library as a RXR-interacting protein in a yeast two-hybrid screen.¹⁷ Full-length SMRT encodes 1495 amino acids, and a possible splicing variant of SMRT was identified and cloned by using unliganded TR as bait, and was termed T3 receptor-associated cofactor-1 (TRAC-1).⁸⁷ TRAC-1 lacks the N-terminal repression domain of SMRT and thus acts like a SMRT truncation mutant (C-SMRT) that can reverse transcriptional repression by unliganded TR and RAR.^{18,87} These studies suggest that transcriptional repression by unliganded receptors may be regulated by combinations of positive and negative corepressor variants. N-CoR was originally identified as a TR-interacting protein in a yeast two-hybrid screen,⁵⁰ which appears to be a full-length version of a RXR-interacting protein named RIP13.⁹¹ The search for full-length RIP13 resulted in identification of a clone (named RIP13a), which encodes a protein similar in structure to SMRT.⁹² N-CoR was also identified in a yeast two-hybrid screen by using Rev-Erb as bait.¹²⁷ Both SMRT and the human N-CoR were also identified in a yeast two-hybrid screen for proteins that interact with the acute promyelocytic leukemia fusion protein, PML-RAR (Chen and Evans, unpublished data).

SMRT and N-CoR are distinct from other identified corepressors (see Ref. 54). Intriguingly, these two proteins are related (Figure 2), as first suggested by the sequence similarity between the C-terminus of SMRT and the polypeptide encoded by RIP13.¹⁷ Comparison of SMRT with N-CoR indicates that these two proteins share 41% identity over the entire SMRT sequence and that N-CoR contains a unique N-terminal extension of about 1000 amino acids.¹⁸ The similarity is more apparent at the N-terminal transcriptional repression domain and the C-terminal receptor-interacting domains, suggesting that SMRT and N-CoR are members of a new family of receptor-associated corepressors.

A. Evidence that SMRT/N-CoR are Transcriptional Corepressors

Several pieces of evidence establish SMRT and N-CoR as receptor-associated corepressors.

SMRT and N-CoR interact efficiently with unliganded TR and RAR and dissociate from the receptors upon ligand binding.^{17,50} SMRT and N-CoR also interact with other transcriptionally repressive receptors, including COUP-TF1,⁹³ Rev-Erb, RVR,^{27,127} and antagonist-bound ER and PR,^{52,95} as well as the oncogene v-erbA and the RAR dominant-negative mutant RAR403.^{17,87} Mutations that block transcriptional repression activities of the receptors also impair the abilities to interact with SMRT and N-CoR. Furthermore, SMRT and N-CoR contain strong transcriptional repression domains,^{69,79} and overexpression of full-length SMRT reinstates transcriptional repression blocked by unliganded RAR and TR LBDs whereas overexpression of the receptor-interacting domain of SMRT antagonizes receptor-mediated repression. Consistent with their regulatory roles in transcription, both SMRT and N-CoR are exclusively nuclear.^{18,69,97} Together, these studies

indicate that SMRT and N-CoR are transcriptional corepressors for nuclear receptors.

However, most of the evidence that supports the corepressor function of SMRT and N-CoR are based on chimeric systems such as Gal4 DBD fusions, which in most cases is more sensitive for analysis of transcriptional repression. The effects of SMRT and N-CoR on natural and hormone-regulated promoters is less clear, although attempts have been made to address this question.^{69,89,97,99,128} In transient transfections, wild-type SMRT potentiates transcriptional repression mediated by a Gal4 DBD-TR fusion protein¹²⁸ as well as from natural promoters or response elements linked to luciferase reporter.⁶⁹ However, only about two- to threefold further repression is observed, perhaps due to the already low level of basal transcription. Transiently transfected N-CoR also potentiates repression mediated by Gal4 DBD-TR fusion and RevErb

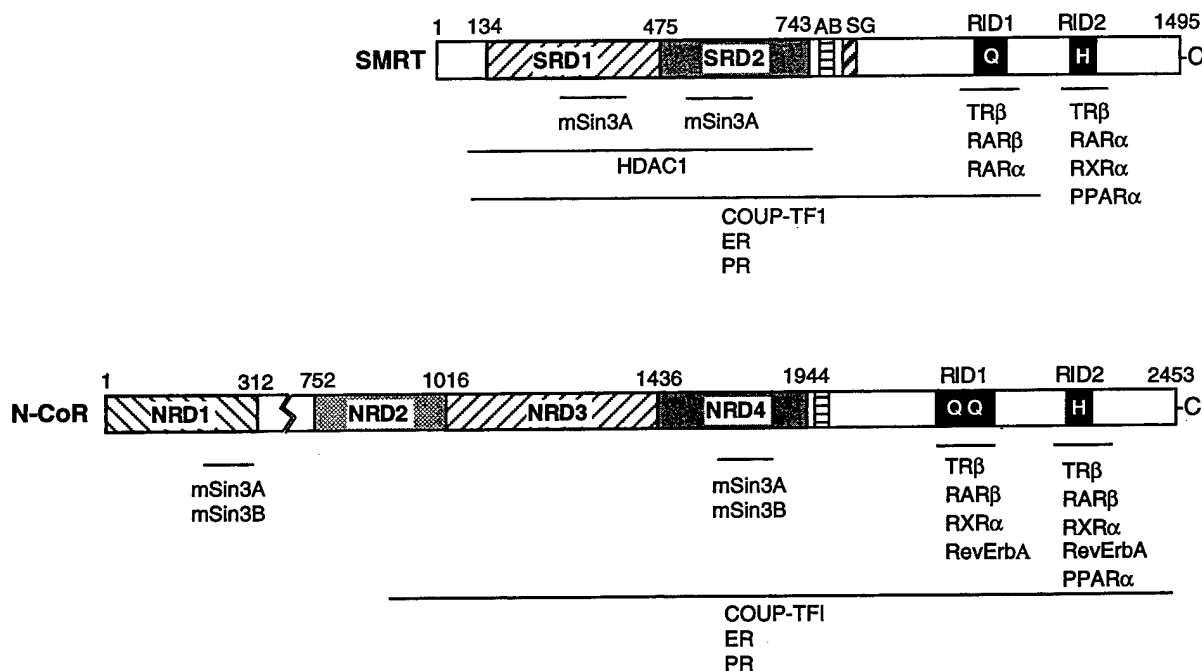


FIGURE 2. The SMRT/N-CoR family of receptor-associated corepressors. Two SMRT repression domains (SRD1 and SRD2) are located at the N-terminal region. An alternate acidic-basic domain (AB) and a serine-glycine region (SG) are shown. Two independent receptor interacting domains (RID1, corresponding to a glutamine-rich [Q] region; and RID2, corresponding to a helical region [H]) are also shown. The receptors that interact with different regions of the corepressors are shown. Also, the Sin3 interacting domains and HDAC1 interacting domain are indicated.

from a RevDR2 response element, consistent with the ability of N-CoR to associate with RevDR2-bound RevErb.¹²⁸ Similarly, transiently transfected N-CoR was also shown to enhance repression from a DR1 element, albeit, only at a low concentration of transfected plasmid DNA. At high concentrations, N-CoR actually enhances reporter gene expression,⁹⁷ possibly by titrating out other components required for transcriptional repression, and thus stimulating basal promoter activity.⁹⁷ A similar effect was observed on both DR1 and DR5 elements, despite the fact that these two DNA response elements affect N-CoR/receptor interactions in different ways.⁶¹

Further evidence supporting the roles for SMRT and N-CoR in gene regulation involves the effects of the corepressors on genes that are suppressed by ligands for nuclear receptors.^{89,99} It has been known for more than a decade that thyroid hormone can repress, as well as activate, nearly equal numbers of genes.⁸² The best-studied examples include the hypothalamic thyrotropin-releasing hormone (TRH) and the pituitary thyroid-stimulating hormone α - and β -subunit (TSH α and TSH β) genes, which are subject to feedback inhibition by T3. The promoters of these negatively regulated genes are sufficient to confer T3-dependent repression, and overexpression of TR was shown to activate rather than repress basal transcription even in the absence of ligands.^{15,46} Overexpression of SMRT and N-CoR stimulates, rather than represses, basal promoter activities of these promoters.⁹⁹ Further, the DNA-binding domain of TR is not required for this corepressor-dependent activation. Therefore, the corepressor-TR complex may be recruited to local promoters via other DNA-binding proteins.⁹⁹ If this scenario is correct, then the TR-corepressor complex would stimulate, rather than repress, transcription from such negative promoters. Because no evidence has been provided to support the recruitment of the TR-corepressor complex to the promoters, this model remains speculative, and response elements have yet to be identified in these promoters. It remains possible that a TR/corepressor-mediated repression of an unknown transcriptional repressor(s) may mediate the inhibition of TRH and TSHs expression. Further studies will be

required to better understand mechanisms that nuclear receptors and corepressors utilize to enhance transcription of genes that are repressed by thyroid hormone.

B. Interaction Domains between SMRT/N-CoR and Nuclear Receptors

The hinge region of TR was originally shown to interact directly with a putative helical region in N-CoR,⁵⁰ while further analysis of TR revealed a major contribution of the C-terminal region for efficient association with SMRT.⁶⁹ Nested deletional analyses suggest that helix 11 of the TR LBD plays an important role in stabilizing SMRT association, presumably by collaborating with the N-terminal helix 1-2 region.⁶⁹ The interaction of SMRT with either the N terminal or C terminal portions of the LBD alone is weak but detectable, suggesting that these two interacting surfaces may synergize with each other to promote protein-protein interaction that ensures appropriate recruitment of the corepressors. Likewise, two independent TR regions act synergistically for interaction with N-CoR.^{27,92,127} It has recently been shown that a receptor dimer is required for interaction with SMRT and N-CoR, and SMRT/N-CoR may contribute differentially to receptor-specific transcriptional repression.¹²⁸ Furthermore, an antagonist to transcriptional activation by RXR homodimer promotes association with the corepressor SMRT.⁶⁴ These studies suggest that SMRT and N-CoR may utilize similar but distinct mechanisms for interaction with nuclear receptors.

Two distinct subdomains in SMRT are capable of interacting independently with nuclear receptors. Interestingly, these two receptor-interacting domains interacted differently with TR and RAR.⁶⁹ The N-terminal RID-1 region of SMRT interacts better with RAR and contains a glutamine-rich domain, whereas the C-terminal RID-2 region interacts better with TR and contains a putative helical domain. The differential receptor-interacting properties of these two domains suggest that SMRT/N-CoR may utilize distinct mechanisms for binding to different receptors. Because the RID-2 region of N-CoR has

been shown to interact directly with the "hinge" region of TR,⁵⁰ the N-terminal RID-1 region might possibly interact with the C-terminal region of the LBD.

The ability of hormones to activate a given nuclear receptor depends on the presence of a C-terminal activation region known as τ c, τ 4 or AF2-AD, which functions as a ligand-dependent activation domain in the context of an intact LBD or as an autonomous activation element when fused with Gal4 DBD (for review, see Refs. 49,83). Previous studies have shown that this AF2-AD helix is also required to relieve repression by corepressor dissociation,⁵ presumably due to a conformational change of this helix before and after hormone binding. Indeed, comparison of the X-ray crystal structures of unliganded RXR⁸ with liganded TR¹¹¹ and RAR⁸⁵ strongly indicate that the AF2-AD helix (helix 12 in LBD) may undergo a drastic positional shift upon hormone binding. Consequently, deletion of the AF2-AD domain from either TR or RAR results in constitutive repression,^{23,24} which might come from the inability of truncated receptors to release SMRT and N-CoR.^{17,18,50} Presumably, the ligand-induced positional shift in the AF2-AD helix may mask the surface of the core LBD that is critical for interaction with corepressors. Alternatively, the AF2-AD may induce a conformational change in the LBD that disrupts corepressor-receptor interaction.

Recently, the role of τ 4/ τ c/AF2-AD in the release of repression and transcriptional activation was further investigated by using the repression core of RAR fused to heterologous activation domains.⁶ A 9-aa portion of the TR β AF2-AD is sufficient to restore the ligand-dependent activation by the RAR403 dominant-negative mutant. A similar effect is observed by using activation domains from transcription factors other than members of the nuclear receptor superfamily. However, not all activation domains tested induce ligand-dependent transactivation when fused to RAR403, suggesting a structural constraint for the ability of ligand to regulate the activation domain function. Further, activation function of the TR AF2-AD is not required for ligand-dependent release of repression. Surprisingly, dissociation of SMRT and N-CoR is also not required for

ligand-dependent activation restored by the heterologous ADs, as evidenced by both yeast two-hybrid assays and gel mobility shifts.⁶ Considering the role of SMRT and N-CoR on wild-type TR and RAR, these studies suggest that the heterologous AD may inactivate corepressor function and that displacement of corepressors is a subsequent step that is not absolutely required to relieve repression. It is possible that, for wild-type TR and RAR, the inactivation mechanism includes the displacement step in order to assure a complete absence of repression function in the activation complex. To fully understand corepressor dissociation upon ligand binding, it will be necessary to investigate the exact structural changes on the receptor before and after hormone binding, as well as the exact interactions between corepressors and unliganded receptors.

Both SMRT and N-CoR contain strong transcriptional repression activity that can be transferred to a heterologous DNA-binding domain.^{17,18,50} Two independent repression domains initially found at the N-terminal of N-CoR are not present in SMRT.⁵⁰ However, a strong repression activity was found in the N-terminal 981 amino acids of SMRT.¹⁷ Further mapping studies revealed that this N-terminal region of SMRT actually contains two independent repression domains called SMRT repression domains (SRD).^{69,79} Because both SRD-1 and SRD-2 are highly conserved with corresponding regions in N-CoR sequences (44 and 47% identities, respectively), it was not surprising that the corresponding regions in N-CoR also confer strong repression. In all, N-CoR contains four independent repression domains, termed N-CoR repression domain 1–4 (NRD-1 to NRD-4). Some of these repression domains can repress basal transcription as efficiently as the full-length protein, suggesting that multiple repression domains may act redundantly to ensure appropriate repression. Sequence comparison of these repression domains provides little information regarding possible mechanisms of repression. However, within SRD-1 and the corresponding NRD-3, four potential repeated motifs sharing a consensus sequence of GSITQGTPA have been identified.⁹² In addition, two other potential repeats with a consensus sequence of KGHVI•YEG were noted. These motifs are well conserved between SMRT

and N-CoR, suggesting that they might contribute to the repression activity of this domain.

C. Interactions between mSin3 and SMRT/N-CoR

Several recent reports demonstrated that SMRT and N-CoR could associate with a cellular complex containing mSin3 and histone deacetylases, suggesting that histone deacetylation could be a mechanism of transcriptional repression mediated by SMRT/N-CoR and unliganded receptors.^{1,25,42,44,55,63,79,84,116} The acetylation state of core histones in the nucleosome has long been postulated to be involved in transcriptional regulation (see Refs. 9,117). Hyperacetylation of histones at the promoter region results in decondensation of chromatin, thereby increasing the accessibility of transcription factors. This process is correlated with gene activation and is consistent with recent findings that transcriptional coactivators like CBP/p300 and their associated protein P/CAF are histone acetyltransferases.¹²¹ Conversely, histone deacetylation is thought to reestablish a condensed chromatin structure, thereby restricting access of general transcription factors. In support of this hypothesis, a yeast corepressor RPD3 was found to act as a histone deacetylase and to assist transcriptional silencing of several yeast genes.^{101,108} Genetic experiments further suggest that yRPD3 acts in the same pathway with the yeast transcriptional repressor Sin3, because both mutations lead to derepression of the same set of genes.¹⁰⁸ These and other studies have led to the suggestion that certain DNA-binding transcriptional repressors interact with Sin3, which in turn recruits histone deacetylases such as RPD3. The final result of these recruitment events is chemical modification of histones, chromatin condensation, and transcriptional repression.^{110,115}

Two mammalian homologs of yeast Sin3 (mSin3A and mSin3B) were identified and found to function as transcriptional corepressors for Mad/Mxi-mediated repression.^{4,88} In addition, two mammalian homologs of the yeast RPD3 (called HDAC1, formally HD1, and mRPD3) were cloned and shown to act as histone deacetylases and transcriptional repressors when

fused to heterologous DBD.^{101,120} mSin3 and HDAC associate with each other in a cellular complex that can be coimmunoprecipitated by specific anti-N-CoR antibodies. Immunoprecipitation of mammalian whole cell extracts by anti-mSin3A, mSin3B, or mRPD3 revealed a cellular complex containing N-CoR,⁴⁴ suggesting that N-CoR can associate with mSin3 and RPD3. Coimmunoprecipitation using purified N-CoR protein detected a direct interaction with mSin3A and mSin3B but not with mRPD3. However, anti-mSin3A and anti-mSin3B antibodies immunoprecipitated mRPD3. Coordinately, a substantial histone deacetylase activity was detected in immunoprecipitate by anti-N-CoR antibodies. Together, these data suggest a cellular complex containing N-CoR, Sin3A and B, and mRPD3. Similarly, it was demonstrated that SMRT can associate with mSin3A and form a complex with HDAC1, suggesting that SMRT is also part of a corepressor complex containing mSin3 and histone deacetylases.⁷⁹ These studies suggest that histone deacetylation may be a mechanism of transcriptional repression by unliganded nuclear receptors. They also suggest that the corepressor complex is heterogeneous, containing different subunits of corepressors (SMRT, N-CoR, or their splicing variants), co-corepressors (mSin3A or mSin3B), and perhaps different histone deacetylases (HDAC1 or mRPD3).

A yeast two-hybrid screen for mSin3A-PAH1 domain-interacting proteins identified a splicing variant of N-CoR containing two stretches of the N-CoR sequence.¹ *In vitro* GST pull-down assays revealed that an N-CoR fragment (N-SID^{PAH1}) between amino acids 1681 to 1893 within the NRD-4 domain is sufficient for interaction with a short splicing form of mSin3B (mSin3BSF), which contains only PAH1 and PAH2 domains.¹ Similarly, an N-CoR fragment between amino acids 1829 and 1940 is sufficient for interaction with the PAH1 region of both mSin3A and mSin3B.⁴⁴ Together, these data delineated an N-CoR fragment between aa 1829 and 1893 that is critical and perhaps sufficient for mediating protein-protein interactions with the PAH1 domain of mSin3A and mSin3B. This interacting surface defines the first contacting point between these two proteins. In addition, disruption of the presumed α -helices

A and/or B of mSin3B PAH1 domain by proline substitution was shown to abolish interaction with N-CoR,¹ suggesting that the interaction between N-SID^{PAH1} and PAH1 domains may be mediated through a helical structure.

Immunoprecipitation experiments using full-length N-CoR and the long form of mSin3B with proline substitution within the helix A of PAH1 domain indicated the existence of an additional interacting surface between N-CoR and mSin3.¹ The second N-SID domain is localized between amino acids 254 and 312 (called N-SID^{PAH3}) and appears to mediate interactions with PAH3 and part of the linker region between PAH3 and PAH4 of both mSin3A and mSin3B.⁴⁴ N-SID^{PAH3} is located within the first N-CoR repression domain (NRD-1). These studies are consistent with the ability of N-SID^{PAH1} and N-SID^{PAH3} fragments to repress basal transcription,⁴⁴ suggesting that recruitment of mSin3A or B may be sufficient for transcriptional repression by N-CoR. On the other hand, mutations that disrupt helix A of the PAH1 domain in mSin3B_{SF}, which inhibits its interaction with N-CoR, also attenuate the transcriptional repression activity of mSin3B.¹ Together, these data suggest that mSin and N-CoR may depend on each other for transcriptional repression, and therefore may exist as a corepressor complex. The interactions between mSin3B and N-CoR have also been demonstrated in mammalian two-hybrid and far-Western analysis.⁶⁹ The interaction between N-SID^{PAH3} and PAH3 domain therefore defines the second point of contact that brings these two proteins together. Intriguingly, no interaction between mSin3 and the second or third repression domains of N-CoR (NRD-2 and NRD-3) could be detected.^{44,69} However, these two domains also confer strong autonomous transcriptional repression to Gal4 DBD.^{44,50,69} These results suggest additional mechanisms of transcriptional repression by N-CoR and that a single corepressor may utilize, perhaps simultaneously, multiple mechanisms for transcriptional repression. Consistent with this, some strong repressors do not appear to interact with mSin3 or histone deacetylases.⁴⁴ Furthermore, Ssn6/Tup1-mediated repression does not require histone deacetylation,⁵⁵ further supporting the idea of multiple pathways leading to transcriptional repression.

Similarly, a direct interaction between mSin3A and SMRT has been demonstrated by both GST pull downs and far-Western analyses, as well as by the two-hybrid assay *in vivo*.^{69,79} A SMRT fragment corresponding to SRD-1 was capable of interaction with mSin3A in a GST pull-down assay, and both SRD-1 and SRD-2 fragments are capable of bringing down mSin3A and HDAC1.⁷⁹ Further analysis indicated that, unlike N-CoR, SMRT was not able to interact with mSin3B and that all four PAH domains in mSin3A seem to be required for efficient interaction. On a far-Western blot, we found that mSin3A interacts most efficiently with SRD-2 and the corresponding NRD-4 domains.⁶⁹ It is currently unclear whether a similar double-contact as seen with N-CoR and mSin3 also exists between SMRT and mSin3A. That NRD-4 and SRD-2 interacted similarly with mSin3A in a far-Western blot suggests that these two related repression domains might interact with the same region of mSin3.

D. Recruitment of Histone Deacetylases by the Corepressor Complex

The two mammalian histone deacetylases, HDAC1¹⁰¹ and mRPD3,¹²⁰ have been shown to associate with mSin3A and mSin3B in a cellular complex.^{1,42,63} These data suggest that interaction between SMRT/N-CoR and mSin3A/B may result in recruitment of HDACs. Indeed, it was demonstrated in a GST pull-down assay that GST-SRD fusions were capable of retaining both mSin3A and HDAC1.⁷⁹ A two-hybrid interaction test in mammalian cells also suggests an interaction between VP-SMRT and Gal-HDAC1 fusions⁷⁹ and between VP-mSin3A and Gal-HDAC1.⁶⁹ However, several attempts to detect direct interactions between N-CoR and mRPD3, mSin3A/B and mRPD3, or SMRT and HDAC1 were unsuccessful, suggesting that the recruitment of histone deacetylase may require additional intermediate factors. Coordinately, at least five more polypeptides were found in the immunoprecipitate by anti-mSin3 antibody,⁴² suggesting that some of these proteins may bridge the interaction between SMRT/N-CoR/Sin3 and HDAC.

In vitro and *in vivo* data all suggest that both SMRT and N-CoR can exist in a cellular complex containing mSin3A/B and HDAC1/mRPD3. Inhibition of individual subunits of this putative complex by microinjection of specific antibodies suggests that this corepressor complex plays an essential role in transcriptional repression by both unliganded receptors and Mad/Mxi complex.⁴⁴ Therefore, this multiprotein corepressor complex could be an integrated unit that negatively controls transcription by different transcriptional repressors involved in diverse signaling processes. However, this putative "negative integrator" appears to display functional specificity for certain repressors but not for all. How multiple, different transcriptional repressors are also assisted by this corepressor complex remains unclear, as does the mechanism of transcriptional repression mediated by the other repressors. Intriguingly, two out of the four identified repression domains in N-CoR do not seem to interact with mSin3A/B or HDAC,^{69,105} even though these domains alone are capable of repressing transcription, apparently through a Sin3/HDAC-independent mechanism. These studies suggest that a repressor molecule may be able to target multiple repression pathways simultaneously, perhaps to ensure appropriate inhibition of target genes. In support of this idea, previous evidence has shown that direct protein-protein interaction with TFIIB or TBP may contribute to transcriptional repression by unliganded TR.^{31,33} Further studies will determine whether these two putative repression pathways both contribute to repression by unliganded receptors, and whether deacetylation by HDAC will result in an altered interaction of TFIIB/TBP with unliganded receptors.

E. The Potential Role of SMRT and N-CoR in Human Disorders

Mutations in members of the nuclear receptor superfamily frequently result in neoplastic and endocrine disorders. One example is the genetic disease characterized by resistance to thyroid hormone syndrome (RTH). Typically, RTH is associated with TR β mutants that interfere with the wild-type receptor function (dominant nega-

tive effect). Characterization of these TR mutants reveals an aberrant association with SMRT, where the corepressor is not dissociated by hormone.¹²⁵ Two of these mutants exhibit approximately wild-type levels of T3 binding, but no ligand-sensitive dissociation of SMRT could be observed, suggesting that hormone binding per se is not sufficient for release of SMRT. Furthermore, these two mutations demonstrate impaired ligand-dependent transcriptional activity and function as constitutive repressors, consistent with the idea that constitutive association with corepressors correlates with transcriptional repression.¹⁷ These studies suggest that altered protein-protein interactions between RTH mutants and SMRT contribute to this endocrine disorder. However, a correlation between the RTH phenotype and a specific altered interaction with SMRT has not been observed. Because RTH is associated with diverse phenotypes, the aberrant association with SMRT may not account for all the observed physical outcomes. Interactions with additional cofactors, such as N-CoR or other as-yet-to-be identified partners, may also contribute to the heterogeneity of this endocrine disorder.

SMRT/N-CoR may also be involved in human acute promyelocytic leukemia (APL), which results from RAR α gene translocation. These translocations create RAR fusion proteins that are believed to be responsible for the oncogenic phenotype of APL. Not surprisingly, one of these RAR fusion proteins (PML-RAR) has been shown to interact with SMRT⁸⁷ (Chen and Evans, unpublished data). The association between SMRT/N-CoR with PML-RAR is ligand-sensitive, correlating with the ability of RA to activate PML-RAR and to induce APL cell differentiation. However, the role of SMRT and N-CoR in the oncogenic activity of PML-RAR is unclear. The interactions between two other APL fusion proteins (NPM-RAR and PLZF-RAR) with SMRT or N-CoR have not been investigated. Because these two APL cases do not respond to RA therapy, their interactions with SMRT/N-CoR, and the effect of ligand on these interactions, may provide insights into the role of corepressors in APL.

Finally, it was reported recently that SMRT and N-CoR can also interact with steroid recep-

tors, including ER, PR, and perhaps GR.^{52,95} In fact, a human N-CoR clone was identified and three different N-CoR isoforms were cloned using PR as bait in the presence of antiprogesterin RU-486. Apparent interactions between hN-CoR and RU486-bound PR or Tamoxifen-occupied ER were observed in the yeast two-hybrid system. These interactions were observed only when receptors were bound to type-II antagonists, and not to type-I antagonists (pure antagonist) such as ZK98299 for PR and ICI16348 for ER. The interactions were also observed *in vitro* by GST pull downs where GST-ER interacts with full-length SMRT in a ligand-insensitive manner;⁹⁵ (Chen, unpublished data). In transient transfections, both SMRT and N-CoR inhibit the partial agonist activity of type-II antagonists like RU-486 on ER, PR, and GR, but have little effect on basal or agonist-stimulated transcription.⁵² In contrast, the partial agonist activity of type-II antagonists could be further enhanced by overexpression of the coactivators SRC1 or L7/SPA (switch protein for antagonist). Furthermore, this coactivator-enhanced activity could be suppressed or compromised by corepressors,^{52,95} suggesting that the ratio of corepressors to coactivators is an important factor that controls the activity of type-II antagonists. This property of corepressors and coactivators may have important clinical implications in therapeutic applications of these antihormones, whose undesired agonistic effects often diminish their clinical benefits.

IV. CONCLUSIONS

Many important developmental and physiological processes are mediated through the actions of steroid and thyroid hormones that bind to their respective nuclear receptors that regulate specific sets of gene expression. The identification and cloning of coregulatory molecules for nuclear receptors has provided additional layers of complexity and excitement, not only in understanding the exact mechanisms of hormone action, but also potentially in gaining control over physiological and phenotypic responses associated with hormones. The current flood of publica-

tions in this field has made it impossible to cover all aspects of these coregulatory molecules. We have presented our discussion mainly in the action of three related nuclear receptor coactivators and two related nuclear receptor corepressors. The current evidence strongly indicates that SMRT/N-CoR regulate repression of receptor target genes in the absence of hormone, and upon hormone treatment, the SRC coactivators replaces the corepressors and regulate activation of the target genes (Figure 3). Many detailed studies are still needed to further understand the physiological significance of the actions of these coactivators and corepressors. For example, the properties of full-length coactivators and corepressors have not been fully investigated. Such studies will be critical for understanding the role of these cofactors *in vivo*. It is important to note that endogenous, full-length N-CoR remains tightly associated with the liganded receptors.^{25,50,61} However, if a large percentage of the recombinant receptors did not bind ligand or failed to undergo appropriate conformational change after ligand binding, a high background of ligand-independent interactions between N-CoR and the receptor is likely to mask ligand-sensitive interactions in this assay. Consistent with this speculation, recent studies show that prokaryotes lack an efficient cotranslation folding capacity, but most of the protein translated in reticulocyte lysates folds properly.⁸⁰

To add an additional twist of complexity, liganded RAR is capable of interacting simultaneously with both coactivator and corepressor *in vitro*.³⁷ Possibly, the repressive activity of SMRT/N-CoR is dominant over activation by SRC when both are recruited to a DR1 element by the RAR/RXR heterodimer.²⁵ Intriguingly, several RAR chimeras containing a heterologous activation domain can retain SMRT and yet still permit ligand-dependent transcriptional activation.⁶ The ability of RAR-AD chimeras to activate transcription and to retain SMRT suggests that either activation is dominant over repression, or that ligand binding may inactivate the corepressor first, leaving release of corepressor as a subsequent step.⁶ It is also unclear whether more members of the corepressor and coactivator family exist. The fact that a single receptor can interact with multiple cofactors (positive or negative) and that a cofac-

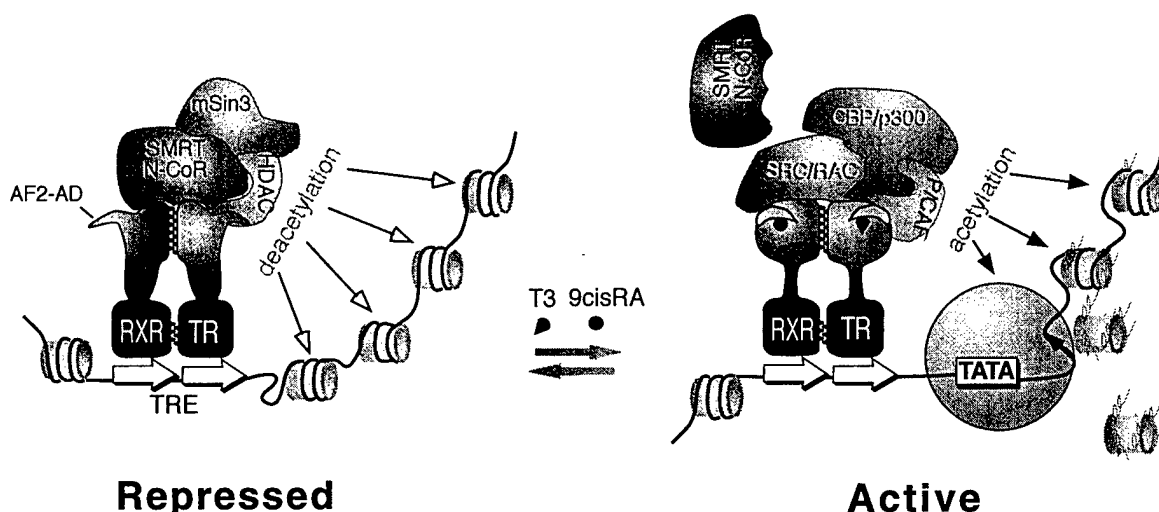


FIGURE 3. Model of the mechanisms of coactivation and corepression in receptor signaling. In the absence of hormone, DNA-bound unliganded receptors recruit SMRT/N-CoR corepressors that target mSin3 and histone deacetylases (HDAC) to catalyze the removal of acetyl group from histones, leading to the generation of a condensed and repressed chromatin structure. Hormone binding induces conformational changes of both receptors that release corepressors and recruit a coactivator complex containing SRC/RAC family proteins and CBP/p300 as well as p300/CBP-associated factor p/CAF. This coactivator complex catalyzes the acetylation of histone, which disrupts nucleosome array, leading to an open and active chromatin structure.

tor can interact with multiple receptors complicate the investigation on the physiological role of these cofactors. Are these families of coactivators and corepressors functionally redundant and perhaps interchangeable *in vivo*? Differences in the function of these two corepressors have been found. For example, N-CoR but not SMRT was found to interact with Rev-Erb on DNA; thus, presumably, N-CoR plays a more important role in mediating silencing by Rev-Erb than SMRT.¹²⁸ Nonetheless, current evidence suggests that both N-CoR and SMRT are silencing mediators for both TR and RAR, and therefore a certain overlap of biological functions should be expected.

Despite these questions regarding the biological function of the coactivators and corepressors, the mechanisms through which these cofactors control transcription have been explored recently. One mechanism that SMRT and N-CoR utilize to repress transcription is linked to histone deacetylation. On the other hand, the SRC coactivators recruit additional coactivators and histone acetyltransferases. These new players apparently provide a direct connection between the action of nuclear receptors and modi-

fication of chromatin structure. Therefore, the mechanisms of transcriptional regulation by nuclear receptors appear to circle around chromatin structure. Perhaps the DNA-bound receptors can conduct both repression and activation without leaving the promoter. However, many studies suggest histone acetylation and deacetylation are not the only stories about repression and activation.^{103,118} Apparently, additional studies are required for further understanding the mechanisms of transcriptional repression and activation. Nevertheless, these recent studies not only open a new door for investigating the exact mechanisms of transcriptional regulation by nuclear receptors, but also provide an excellent opportunity for developing new therapeutic strategies that may contribute to the treatment of human diseases.

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Steroid/Nuclear Receptor Coactivators

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In higher eukaryotes, steroids/thyroid hormones and many lipophilic compounds regulate cellular physiology through binding to the steroid/nuclear receptor proteins. Steroid/nuclear receptors are ligand-dependent transcriptional activators that can stimulate gene expression. This transcriptional activation plays a pivotal role in hormone-regulated physiological and pharmacological responses. In recent years, several steroid/nuclear receptor cofactors have been identified and found to interact with the receptor and modulate

its transcriptional activity. Among these cofactors, a family of three coactivators has been the focus of intense studies. Although gaps remain, progress has been made in understanding how a given coactivator interacts with the receptor and promotes transcriptional activation. We are beginning to understand coactivator action; for instance, several studies have established the molecular basis of antagonism by anti-hormones and the connection of coactivators with human cancers. © 2000 Academic Press.

I. INTRODUCTION

Lipophilic steroids, including estrogen, progesterone, androgens, glucocorticoid and mineralocorticoid, thyroid hormones, retinoids, vitamin D₃, and peroxisome proliferators regulate diverse biological activities including cell proliferation, differentiation, development, and homeostasis. The activities of these compounds are thought to be mediated by members of the steroid/nuclear receptor superfamily, most of which are ligand-regulated transcriptional activators (Mangelsdorf *et al.*, 1995; Mangelsdorf and Evans, 1995; Kastner *et al.*, 1995; Thummel, 1995; Beato *et al.*, 1995). A distinct domain structure, including an N-terminal DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD) characterizes the steroid/nuclear receptors. The DBD binds to specific DNA sequences located within promoter regions of target genes. The LBD binds to specific ligand, which in turn controls the receptor's transcriptional activity by triggering conformational changes in the receptors that affect protein-protein interaction and transcriptional activation. Thus, lipophilic nuclear hormones display diverse biological effects owing to transcriptional activity driven by steroid/nuclear receptors.

The steroid/nuclear receptor assumes both active and inactive conformations depending on ligand binding to the receptor's LBD. The LBD mediates not only ligand-binding, but also protein-protein interaction, transcriptional activation, and transcriptional repression. Located near the C terminus of the receptor, the conserved AF-2 helix domain (also known as AF2-AD, τ C, or τ 4) plays a crucial role in regulating ligand-dependent transcriptional activity. Several lines of investigation have helped elucidate receptor-driven transcriptional activation (Beato *et al.*, 1995). Notably, squelching effects between different receptors provide evidence that cofactors regulate receptor-mediated transcriptional activity (Meyer *et al.*, 1989; Barettino *et al.*, 1994). The identification of receptor-associated proteins involves both genetic and bio-

chemical approaches such as the yeast two-hybrid screen and far-Western-based expression screening (Seol *et al.*, 1995; Cavaillès *et al.*, 1994; Halachmi *et al.*, 1994; Kurokawa *et al.*, 1995; Eggert *et al.*, 1995; Chen and Evans, 1995; Horlein *et al.*, 1995).

The two important classes of steroid/nuclear receptor cofactors are the transcriptional corepressors and coactivators. Corepressors interact with unliganded receptors to inhibit target gene expression. The silencing mediator for retinoid and thyroid hormone receptors (SMRT) and the nuclear receptor corepressor (N-CoR) are examples of the corepressors (Chen and Evans, 1995; Horlein *et al.*, 1995). In contrast, coactivators interact with liganded receptors to enhance transcriptional activation. Many potential coactivators have been identified (Horwitz *et al.*, 1996; Glass *et al.*, 1997; Chen and Li, 1998), including transcriptional intermediate factor 1 (TIF1) (Le Douarin *et al.*, 1995), receptor interacting protein 140 (RIP140) (Cavaillès *et al.*, 1995), androgen receptor activator 70 (ARA70) (Yeh and Chang, 1996), and steroid receptor coactivators (SRCs) (Oñate *et al.*, 1995; Takeshita *et al.*, 1996; Zhu *et al.*, 1996; Li *et al.*, 1997; Anzick *et al.*, 1997; Yao *et al.*, 1996; Chen *et al.*, 1997; Kamei *et al.*, 1996; Torchia *et al.*, 1997). In addition, the general transcriptional coactivators SWI/SNF (Chiba *et al.*, 1994; Khavari *et al.*, 1993; Muchardt and Yaniv, 1993) and CREB/E1A-binding protein (CBP/p300) (Arany *et al.*, 1994; Chrivia *et al.*, 1993; Kwon *et al.*, 1994) enhance transcriptional activation by steroid/nuclear receptors. The role of ligand is to induce corepressor dissociation and coactivator recruitment. Such exchange of corepressors and coactivators on DNA-bound receptors is thought to underscore the mechanism of ligand-dependent transcriptional activation (Fig. 1).

Among the steroid/nuclear receptor coactivators, the SRC family has been the focus of recent intense studies. Compelling evidence suggests that SRC coactivators regulate the transcriptional activity of many steroid/nuclear hormone receptors. Extensive investigations have detailed SRC-receptor interactions at the molecular level and described the mechanism of SRC-regulated transcription. Inactivation of one SRC coactivator in mouse demonstrated that this coactivator is required for maximal hormone responses. In addition, another SRC coactivator is amplified in breast, ovarian, and pancreatic cancers, suggesting an important role for these coregulators in cell growth and differentiation. Understanding SRC coactivators may provide a model system and new insights for therapeutic intervention of hormone-related human diseases. This chapter is intended to summarize recent findings about the function and mechanism of action of the SRC coactivators.

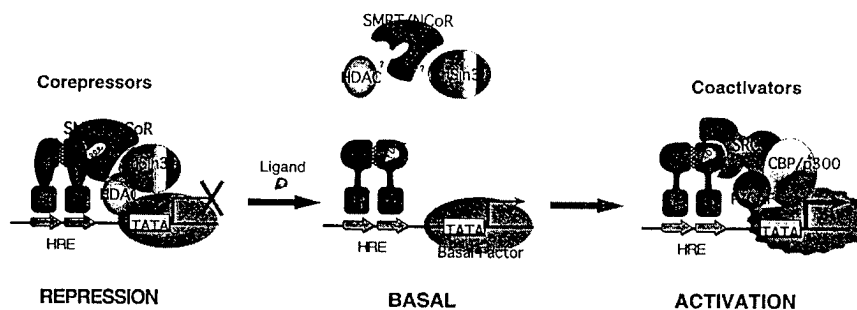


FIG. 1. Model of transcriptional regulation by steroid/nuclear hormone receptors. In the absence of hormone, DNA-bound unliganded receptors recruit nuclear receptor corepressors such as SMRT/NCOR, which target mSin3 and histone deacetylases (HDAC) to catalyze deacetylation of histones, leading to chromatin condensation and transcriptional repression. Hormone binding induces conformational changes of the receptors, resulting in the dissociation of corepressors and recruitment of coactivators, which catalyze acetylation of histones, leading to opening of chromatin and transcriptional activation.

II. IDENTIFICATION OF SRC COACTIVATORS

A. SRC-1

Steroid receptor coactivator 1 (SRC-1), the first member of the SRC family, was isolated in a yeast two-hybrid screen using PR_B-LBD as bait (Oñate *et al.*, 1995). A C-terminal 197-amino-acid (aa) fragment of human SRC-1 was identified that interacts with PR in an agonist-dependent manner (Oñate *et al.*, 1995). Later work identified several isoforms of SRC-1 from both human and mouse libraries as TR, PPAR, or CBP/p300 interacting protein (Takeshita *et al.*, 1996; Kalkhoven *et al.*, 1998; Zhu *et al.*, 1996; Kamei *et al.*, 1996; Yao *et al.*, 1996). RT-PCR analysis confirms two SRC-1 isoforms, SRC-1a and SRC-1e (Kalkhoven *et al.*, 1998). SRC-1e differs from SRC-1a at the C termini; the 1441-aa-long SRC-1a contains 56 unique residues and lacks the most C-terminal 14 amino acids present in SRC-1e (1399-aa) (Kalkhoven *et al.*, 1998), suggesting a potential functional difference between these two isoforms. For simplicity, the full-length SRC-1a will be referred to as SRC-1 unless specified otherwise.

B. SRC-2 AND SRC-3

Following the identification of SRC-1, glucocorticoid receptor interacting protein 1 (GRIP1) was isolated in yeast two-hybrid screen (Hong

et al., 1996, 1997). At about the same time, transcriptional intermediate factor 2 (TIF2) was identified as a 160-kDa human protein that interacts with liganded ER and RAR in a far-Western-based assay (Voegel *et al.*, 1996). Human TIF2 contains 1464 amino acids that are similar to the mouse GRIP1 (over 94% identity), suggesting that TIF2 and GRIP1 are the mouse and human orthologs. In addition, NCoA-2 was reported as a mouse variant of GRIP1 (Torchia *et al.*, 1997). The 1463-aa-long NCoA-2 is nearly identical to GRIP1, except for several single amino acid substitutions and two unrelated gaps at residues 251–320 and 959–982 of GRIP1. In addition, a rat homolog of TIF2 was recently identified as a PPAR α -interacting protein in a yeast two-hybrid screen (Leers *et al.*, 1998). Sequence comparison between GRIP1/TIF2/NCoA-2 and SRC-1 reveals high similarity (Fig. 2), especially in the N-terminal domain, which is related to the bHLH (basic-helix-loop-helix)-PAS (Per-Arnt-Sim) domains in many transcriptional regulators (Swanson *et al.*, 1995; Lindebro *et al.*, 1995; Zelzer *et al.*, 1997). While bHLH-PAS is the most conserved domain among the SRC family members, its function remains undetermined in this coactivator family. Because GRIP1/TIF2/NCoA-2 is an SRC-1-related gene, it will be referred to as SRC-2 unless specified otherwise.

The SRC family was established when p/CIP, RAC3, ACTR, AIB1, TRAM-1, and SRC-3 were cloned and found as the third member of the

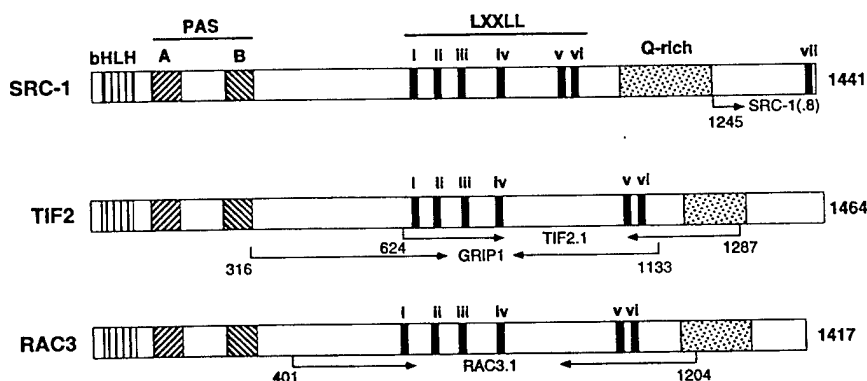


FIG. 2. Schematic representation of the structural domains of SRC family proteins. The N-terminal region contains highly conserved bHLH, PAS A and B domains. The central regions contain six LXXLL motifs (i to vi). SRC-1 contains an additional LXXLL motif at its C terminus. In addition, the C-terminal region contains a glutamine-rich domain, where consecutive glutamine track is present in RAC3 but not in TIF2 or SRC-1. SRC-1 is also known as NCoA-1 or NRC-1. TIF2 is also known as GRIP1 and NCoA-2. RAC3 is also known as p/CIP, ACTR, AIB1, SRC-3, and TRAM-1. The regions encoded by the original clones RAC3.1, TIF2.1, GRIP1, and SRC-1(8) are indicated with arrows.

family (Torchia *et al.*, 1997; Chen *et al.*, 1997; Anzick *et al.*, 1997; Li *et al.*, 1997; Takeshita *et al.*, 1997; Suen *et al.*, 1998). p/CIP was identified as a CBP-interacting protein (Torchia *et al.*, 1997). RAC3 was found as an RAR-interacting protein (Li *et al.*, 1997). ACTR was identified as an hRAR β -stimulatory protein (Chen *et al.*, 1997). AIB1 was isolated as a gene amplified from the long arm of chromosome 20 (Anzick *et al.*, 1997). TRAM-1 was isolated as a TR-interacting protein (Takeshita *et al.*, 1997), while SRC-3 was reported as an ER-interacting protein (Suen *et al.*, 1998). Since p/CIP/RAC3/ACTR/AIB1/TRAM-1/SRC-3 is highly related to SRC-1 and SRC-2, it will be referred to as SRC-3 unless otherwise specified. SRC-3 shared about 46% identity with SRC-2, and about 36% identity with SRC-1. Recently, SRC-3 was also identified in *Xenopus* as an RXR-interacting protein (Kim *et al.*, 1998). The xSRC-3 shares 72% identity with SRC-3, 45% with SRC-2, and 38% with SRC-1, suggesting that xSRC-3 may be the homolog of human SRC-3. Analyses of the chromosomal locations of the three SRC coactivator genes mapped AIB1 to chromosome 20q12, a region amplified in breast cancer (Anzick *et al.*, 1997). The SRC-1 gene was mapped to chro-

TABLE I
SYNONYMS OF SRC COACTIVATORS

| | Species | Genbank | References |
|---------|----------------|---------------|--------------------------------|
| SRC-1 | | | |
| SRC-1 | Human | U90661/U40396 | Oñate <i>et al.</i> (1995) |
| F-SRC-1 | Human | U59302 | Takeshita <i>et al.</i> (1996) |
| hSRC-1a | Human | AJ000881 | Kalkhoven <i>et al.</i> (1998) |
| SRC-1e | Human | AJ000882 | Kalkhoven <i>et al.</i> (1998) |
| mSRC-1 | Mouse | U64828 | Yao <i>et al.</i> (1996) |
| mSRC-1a | Mouse | U56920 | Kamei <i>et al.</i> (1996) |
| mNRC-1 | Mouse | U64606 | Zhu <i>et al.</i> (1996) |
| SRC-2 | | | |
| TIF2 | Human | X97674 | Voegel <i>et al.</i> (1996) |
| rTIF2 | Rat | AF000582 | Leers <i>et al.</i> (1998) |
| GRIP1 | Mouse | U39060 | Hong <i>et al.</i> (1997) |
| NCoA-2 | Mouse | AF000582 | Kamei <i>et al.</i> (1996) |
| SRC-3 | | | |
| RAC3 | Human | AF010227 | Li <i>et al.</i> (1997) |
| SRC-3 | Human | | Suen <i>et al.</i> (1998) |
| AIB1 | Human | AF012108 | Anzick <i>et al.</i> (1997) |
| ACTR | Human | AF036892 | Chen <i>et al.</i> (1997) |
| TRAM-1 | Human | AF016031 | Takeshita <i>et al.</i> (1997) |
| p/CIP | Mouse | AF000581 | Torchia <i>et al.</i> (1997) |
| xsRC-3 | <i>Xenopus</i> | AF044080 | Kim <i>et al.</i> (1998) |

mosome band 2p23 and TIF2 was mapped to 8q21.1 (Kalkhoven *et al.*, 1998), where no amplification in breast cancer was found. Table I lists SRC coactivators synonyms.

C. STRUCTURAL DOMAINS OF SRC COACTIVATORS

Each SRC coactivator contains an N-terminal bHLH domain found in many transcriptional regulators (Fig. 3). The bHLH domain can act as a DNA-binding and/or dimerization interface in several transcription factors (Murre *et al.*, 1989a,b). Immediately adjacent to the bHLH motif is a region similar to the PAS domain found in Period (Per), Aryl hydrocarbon receptor (AhR), the AhR nuclear translocator protein (Arnt), and single-minded (Sim). The PAS domain is present in a class of proteins involved in regulation of *Drosophila* development and cellular signaling induced by hypoxia and dioxin treatment. Interestingly, the bHLH-PAS domain is the most conserved region within the SRC coactivators, indicating the SRC coactivators belong to a large family of bHLH-PAS-containing proteins. The PAS domains in AhR, Arnt, and Sim play important roles in protein-protein interaction, heterodimeric partner selection, and target gene specificity (Swanson *et al.*, 1995; Lindebro *et al.*, 1995; Zelzer *et al.*, 1997). Although the function of the bHLH-PAS domain in SRC coactivators remains unknown, this region could possibly mediate intra- or intermolecular interaction.

Apart from SRC-1 and SRC-2, the structure of SRC-3 is unique in that it contains consecutive poly-glutamine (poly-Q) tracks that result from expansion of CAG repeats. Expansion of poly-Q track is associated with several human diseases (Koshy and Zoghbi, 1997; Reddy and Housman, 1997; Butler *et al.*, 1998). In SRC-3, two poly-Q tracks are found at both ends of the glutamine-rich domain. At the C-terminal location, three of the five cloned human SRC-3 alleles contain 26 consecutive glutamines, while two of them contain 29 consecutive residues. Consistently, polymerase chain reaction products of the SRC-3 CAG repeats at this location revealed size polymorphism (Shirazi *et al.*, 1998). Interestingly, *Xenopus* SRC-3 contains only four consecutive glutamines at this position. Likewise, the mouse SRC-3 (p/CIP) contains no significant poly-Q track at this site. The corresponding region of SRC-2 contains three and four consecutive glutamines in the mouse and human SRC-2, respectively, while no obvious glutamine repeats are present in SRC-1 at this location. The second poly-Q track is more evident within the mouse SRC-3 (p/CIP), which contains a track of 23 consecutive glutamines at a position around residue 1000 near the N-terminal end of the glutamine-rich domain. This location contains five consecu-

tive glutamines in all five human SRC-3 proteins and four glutamines in the *Xenopus* protein. Conceivably, the relative length and position of these poly-Q tracks may distinguish functional differences among SRC coactivator members and alleles.

III. INTERACTION WITH NUCLEAR RECEPTORS

A. LIGAND-DEPENDENT INTERACTION

Implication of SRCs as transcriptional coactivators for steroid/nuclear receptors first came from the observation that an SRC interacts with a receptor in a ligand-dependent manner, suggesting a role in transcriptional activation. Ligand-dependent interactions of SRCs with steroid/nuclear receptors have been demonstrated with multiple assay systems. Using the yeast two-hybrid assay, all three SRC coactivators have been shown to interact with multiple members of the steroid/nuclear receptor family in a ligand-dependent manner (Oñate *et al.*, 1995; Chen *et al.*, 1997; Li *et al.*, 1997; Voegel *et al.*, 1998; Hong *et al.*, 1997). In addition, coimmunoprecipitation and subcellular colocalization also detect *in vivo*, ligand-dependent interactions of SRC with steroid/nuclear receptors. Specifically, subcellular colocalization has been used to analyze ligand-dependent interaction of TIF2 with RAR, ER, and PR (Voegel *et al.*, 1996). Because a truncated TIF2 mutant (TIF2.1) does not contain a nuclear localization signal and remains in the cytoplasm, it demonstrates ligand-dependent translocation from the cytoplasm into the nucleus via interaction with liganded receptors targeted to the nucleus (Voegel *et al.*, 1996).

The association of two proteins *in vivo* involves the formation of a complex containing other proteins. Therefore, *in vivo* interaction observed in the two-hybrid and coimmunoprecipitation assays is usually insufficient to conclude direct interaction between two proteins. GST pull-down and far-Western assays are commonly utilized for detecting protein-protein interaction *in vitro*. In addition, the far-Western assay is also used for screening interacting clones; in fact, this method identified two mouse SRC-1s and the TRAM-1 clone (Kamei *et al.*, 1996; Yao *et al.*, 1996; Takeshita *et al.*, 1997). Figure 4 shows an example of ligand-dependent interaction between RAC3 and VDR analyzed by far-Western analysis. The ligand-dependent interaction with steroid/nuclear receptors suggests that SRCs are components of a transcriptionally active complex. Consistently, the SRC coactivator does not interact with steroid/nuclear receptors bound to antagonist.

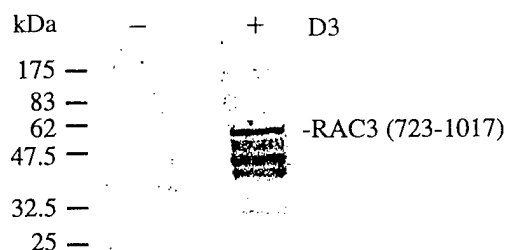


FIG. 4. Ligand-dependent interaction of RAC3 with nuclear receptor. The purified GST-RAC3 (723–1017) fusion protein was separated by SDS-PAGE and analyzed by far-Western analyses for interaction with ^{35}S -labeled hVDR in the absence (–) or presence (+) of $1\ \mu\text{M}$ $1\alpha,25$ -dihydroxyvitamin D_3 . The position of the intact GST-RAC3 (723–1017) fusion protein is as indicated. The smaller peptides that also interact with VDR represent degradation products of GST-RAC3 (723–1017).

B. INTERACTING INTERFACES

The mechanism by which SRC coactivators interact with steroid/nuclear receptors has been extensively analyzed by both biochemical and X-ray crystallography studies. These studies led to the identification of several conserved LXXLL motifs (where L is leucine and X is any amino acid) that are responsible for interaction with liganded receptors and transcriptional activation (Fig. 5). Six LXXLL motifs are located at the central region of all three SRC coactivators and one SRC-1a-unique motif is located at the C terminus (Heery *et al.*, 1997; Li *et al.*, 1997; Torchia *et al.*, 1997). A sequence resembling LXXLL motif has also been identified as receptor-interacting box (NR-box) in TIF1 β (Le Douarin *et al.*, 1996) and in other steroid/nuclear receptor interacting proteins such as RIP140, CBP/p300, and TRIPs (Heery *et al.*, 1997).

The interacting domain between SRC-1 and ER was mapped first by a series of deletion mutants, where aa 570–780 and 1241–1441 of SRC-1a bound ER in an agonist-dependent fashion (Heery *et al.*, 1997; Henttu *et al.*, 1997; Kalkhoven *et al.*, 1998). The aa 570–780 fragment contains three conserved LXXLL motifs (i, ii, iii) and the C-terminal aa 1241–1441 fragment contains one motif (vii), which is present only in SRC-1a but not in SRC-1e or other SRC coactivators. Additionally, other regions of SRC-1a are also capable of binding ER and PR in an AF2- and ligand-independent manner, but the significance of these interactions remains unclear (Oñate *et al.*, 1998; Kalkhoven *et al.*, 1998).

Experiments using site-directed mutants and synthetic peptides have

provided strong evidence for LXXLL motifs in mediating interaction with liganded steroid/nuclear receptors. First, a series of Gal4 DBD fusions with each LXXLL motif (i, ii, iii, or vii) interacts independently with ER in a ligand-dependent manner (Heery *et al.*, 1997), suggesting that an individual motif is sufficient for mediating the interaction. This study suggests that motif ii interacts most tightly with ER, while other unrelated sequences containing similar LXXLL core sequences failed to interact, suggesting that the LXXLL alone is insufficient and that flanking residues are also important for the interaction. Similarly, others observed strong ligand-dependent interactions of motif ii and the C-terminal motif of mouse SRC-1 (NCoA-1) with ER and RAR (Torchia *et al.*, 1997). In support of these observations, replacing the leucine doublet of the C-terminal LXXLL motif of SRC-1a with alanines disrupted the interaction with liganded receptors (Heery *et al.*, 1997).

Motifs

| | | | |
|------|------|-------------|-------------------------|
| i. | RAC3 | (615-631) | SKGHKKLLQLLTCSSDD |
| | TIF2 | (640-651) | SKGQTKLLQLLTTKSDD |
| | SRC1 | (632-643) | SQTSKKLVQLLTTTAAE |
| ii. | RAC3 | (678-695) | LQEKHRILHKLLQNGNSP |
| | TIF2 | (683-699) | LKEKHKILHRLLDSSSP |
| | SRC1 | (683-699) | LTERHKILHRLDQEG.SP |
| iii. | RAC3 | (730-749) | KKKE...NNALLRYLLDRDDPSD |
| | TIF2 | (738-753) | KKKE...NALLRYLLDKDDTKD |
| | SRC1 | (739-757) | KKESKDHQLLRYLLDKDE.KD |
| iv. | RAC3 | (1025-1041) | QNRPLLNSLDDLVGPP |
| | TIF2 | (1051-1067) | QNRQPFSSPDDLCPH |
| | SRC1 | (904-920) | SEDQCISSQLDELLCPP |
| v. | RAC3 | (1045-1061) | EGQSDERALLDQLHTLL |
| | TIF2 | (1071-1087) | ESPSDEGALLDQLYLAL |
| | SRC1 | (924-940) | EGRNDEKALLEQLVSFL |
| vi. | RAC3 | (1069-1084) | LEEIDRALGIPELVNQ |
| | TIF2 | (1093-1108) | LEEIDRALGIPELVSQ |
| | SRC1 | (948-962) | LAELDRAIGIDKLV.Q |
| vii. | SRC1 | (1424-1440) | QTPQAQKSLLQQLLTE |

FIG. 5. The LXXLL motifs of SRC coactivators. The amino acid residues are shown at right in parentheses. The first six motifs are surrounded by highly charged residues and motifs ii, iv, v, and vi were predicted to form α -helical structures.

Because the three LXXLL motifs (i, ii, iii) are sufficient to interact with liganded receptors, the relative contribution and the specificity of each motif become important to understanding the mechanism of coactivator-receptor interaction. Systematic analysis of each LXXLL motif in the context of full-length SRC-1 ϵ protein suggests that loss of individual motif has little effect on the ability of SRC-1 ϵ to bind ER or to enhance its transcriptional activity (Heery *et al.*, 1997). Conversely, mutation of motif ii in combination with motif i or iii, or both, drastically reduces binding to liganded ER, and the ability to enhance ER-mediated transcription in transfected cells. However, combined mutation of motifs i and iii had less effect. When the mutation was generated in the central receptor interacting domain (635–760) of NCoA-1, it appears that mutation of motif ii is sufficient to abrogate interaction with liganded ER and RAR (Torchia *et al.*, 1997). Because disruption of motif ii blocks the function of motif i and iii within the minimal interacting domain of NCoA-1, but not in the full-length SRC-1 ϵ protein, it is conceivable that other interacting surfaces may contribute to stabilizing the interaction. These studies indicate that motif ii of SRC-1 is the preferred site for interaction with liganded ER, while motifs i and iii may contribute to optimal binding and activation of ER in intact cells.

The relative contribution of LXXLL motifs in mediating interaction with different receptors has also been analyzed by peptide competition assay (Heery *et al.*, 1997; Torchia *et al.*, 1997; Darimont *et al.*, 1998). Consistent with mutational studies, motif ii of SRC-1 is most important for interaction with RAR and TR, whereas C-terminal motif vii is most prominent for interaction ER (Heery *et al.*, 1997; Torchia *et al.*, 1997). For instance, an excess of 24-aa oligopeptide encompassing motif ii of NCoA-1 effectively blocked interaction between liganded RAR and NCoA-1 *in vitro*, but a peptide corresponding to motif i was less effective (Torchia *et al.*, 1997). Similarly, an excess 14-aa oligopeptide encompassing the C-terminal motif of SRC-1 blocks interaction between liganded ER and SRC-1 α , but a peptide-containing leucine doublet mutant has no effect (Heery *et al.*, 1997). Also, a 13-aa peptide of GRIP1 motif ii inhibits the interaction between GRIP1 and TR β LBD, while substitution of the leucine residues with alanines eliminates such inhibitory effect (Darimont *et al.*, 1998). Substitution of the leucine residues with phenylalanines also reduced the competition, suggesting that efficient interaction does not simply rely on the hydrophobicity of the LXXLL motif but rather on the stereochemical property of the side chain of leucine (Darimont *et al.*, 1998). Although motif ii of GRIP1 is the preferred sequence for ER binding, motif iii is preferred by GR

(Ding *et al.*, 1998). Another study shows that motif ii of rTIF2 is most critical for binding with PPAR α and TR α , while motif i is the preferred site for RXR β and motif iii is preferred by GR (Leers *et al.*, 1998). These studies suggest that steroid/nuclear receptors may interact with a given SRC coactivator through a precise arrangement of multiple LXXLL motifs.

C. DETERMINANTS OF LXXLL SPECIFICITY

The presence of four different LXXLL motifs that can each interact independently with liganded receptors suggests a requirement for specificity, since it is not likely that all motifs interact simultaneously with a given receptor. In addition, different coactivators compete rather than cooperate for binding to a given receptor (Leers *et al.*, 1998), suggesting coactivator preference for the receptor. Thus, it is important to understand the mechanism of selectivity of LXXLL motifs for specific receptors. Using synthetic chimeric peptide, the preference of GRIP1 motif ii for TR β appears determined by sequences adjacent to the LXXLL core residues. This was shown by a chimeric peptide containing adjacent sequences of motif ii and LXXLL of motif iii, which competes equally well as the intact motif ii for TR β interaction (Darimont *et al.*, 1998). Conversely, a chimeric peptide containing LXXLL of motif ii flanked by sequences adjacent to a VP16 Fxxhh motif competes poorly for TR β interaction. These studies suggest that both the LXXLL residues and the adjacent sequences of motif ii contribute to TR binding. In contrast, a chimeric peptide containing adjacent sequences of motif ii and LXXLL of motif iii competes equally well for GR binding as intact motif iii. Consistently, a chimeric peptide containing motif iii adjacent sequences and motif ii core competes inefficiently with GR binding. Therefore, the LXXLL core can dictate the selectivity of GR for its preference of motif iii over motif ii of GRIP1.

The specificity determinant of LXXLL motifs on transcriptional coactivation by NCoA-1 (SRC-1) has also been analyzed by site-directed mutagenesis and microinjection assay (McInerney *et al.*, 1998). For microinjection assay, a β -galactosidase reporter driven by specific response elements is injected into cell nuclei, along with specific antibody and a rescuing plasmid (McInerney *et al.*, 1998; Torchia *et al.*, 1997; L. Xu *et al.*, 1998; Korzus *et al.*, 1998). The requirement of specific LXXLL motifs of NCoA-1 for transactivation by different receptors was determined by antibody injection to inhibit reporter gene activation, along with a plasmid expressing wild-type or LXXLL mutant of NCoA-1. In this study, injection of anti-NCoA-1 IgG inhibits transactivation by ER,

PR, RAR, TR, and PPAR γ (McInerney *et al.*, 1998), while coinjection of wild-type NCoA-1 reverses the IgG-mediated inhibition completely (Fig. 6). Coinjection of different LXXLL mutants elicits distinct levels of rescue. For instance, wild-type NCoA-1 and the motif i or iii mutants are capable of reversing IgG-mediated transcriptional inhibition. Double mutation of motifs i and iii had no effect on the coactivation function on ER-mediated transcriptional activation. In contrast, mutation of motif ii abrogated the ability to rescue IgG-mediated inhibition. These studies suggest that motif ii of NCoA-1 is sufficient for supporting ER activation, consistent with previous transient transfection and peptide competition studies (Ding *et al.*, 1998; Heery *et al.*, 1997; Kalkhoven *et al.*, 1998). Mutation of motif ii seems to play a more profound effect in the injection assay, but this might be due to a more severe mutation used in the injection assay (LXXLL \rightarrow LAAAA) than in the transfection assay (LXXLL \rightarrow LXXAA). It was also found that PR and PPAR γ require both motifs i and ii, but not iii, while RAR and TR require motifs ii and iii, but not i, suggesting a distinct pattern of LXXLL motif requirement for different receptors. In addition, the LXXLL motif preference by PPAR γ appears to be regulated by ligands. While troglitazone (TGZ; thiazolidinedione)-activated PPAR γ prefers motif ii over i, prostaglandin J2 metabolites (PGJ2) promote an equivalent, partial requirement for both motif i and ii, but indomethacin alters the preference to motif i over ii (McInerney *et al.*, 1998). This specificity appears to depend on amino acids carboxy terminal to the LXXLL core. Consistently, distinct carboxy-terminal amino acids are required for PPAR γ activation in response to different ligands (McInerney *et al.*, 1998). To-

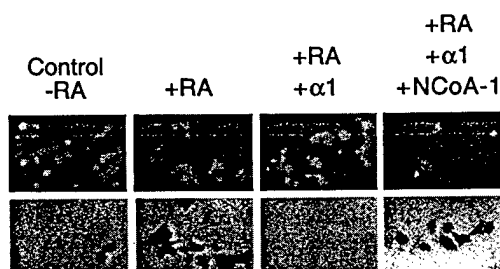


FIG. 6. Probing SRC coactivator function by microinjection assay. Microinjection of affinity-purified anti-NCoA-1 IgG blocked retinoic-acid-dependent activation of the RARE/LacZ reporter. The RA-dependent expression of reporter gene was fully rescued by coinjection of NCoA-1 expression vector. Photomicrographs of rhodamine-stained injected cells and the corresponding protein of X-Gal staining. [Adapted by permission from Fig. 4 of Torchia, J., *et al.* (1997). The transcriptional co-activator p/CIP binds CBP and mediates nuclear receptor function. *Nature* 387, 677–684.]

gether, these studies suggest that LXXLL motifs may serve overlapping roles for both receptor-specific and ligand-specific assembly of a coactivator complex.

The contribution of each LXXLL motif residue for interaction with different receptors has also been analyzed by systemic mutation in combination with microinjection assays (McInerney *et al.*, 1998). The sequences encompassing the eight amino-terminal or carboxy-terminal flanking residues of motif ii of SRC-1 were mutated to alanines and their abilities to restore transactivation by RAR, TR, and ER were analyzed. It was shown that the flanking amino-terminal residues are not essential, while the eight carboxy-terminal residues are required for SRC-1-mediated coactivation on RAR, TR, and ER (McInerney *et al.*, 1998). Additionally, residues +12 and +13 (the first L in LXXLL is designated +1) are required for ER binding, while residues at +6, +7, +11, and +13 are important for interaction with RAR-RXR heterodimer on DNA template. Similar experiments also reveal that amino acids at positions +6, +11, and +13 of motif ii are critical for NCoA-1 binding to TGZ-activated PPAR γ . Intriguingly, when PPAR γ is activated by BRL49653, distinct residues at +8, +9, +10, +12, and +13 become important for NCoA-1 binding. These studies suggest a ligand-specific alteration of receptor structure, which may impose a requirement for different LXXLL residues to achieve high-affinity interactions with the SRC coactivators.

Because many nuclear receptors seem to require two functional LXXLL motifs on one SRC molecule for maximal interaction, it is conceivable that spacing between two motifs may be important for such recognition. Accordingly, deletion of 30 amino acids from the conserved spacing of 50 amino acids between motifs ii and iii severely inhibits the capability of SRC-1 to restore IgG-mediated inhibition on RAR or TR transactivation (McInerney *et al.*, 1998). This deletion does not have any effect on the ability of SRC-1 to rescue PPAR γ function, consistent with observation that motif iii is not essential for SRC-1 coactivation of PPAR γ . However, shortening the spacing between motifs i and ii inhibits the function of SRC-1 to support PPAR γ transactivation, consistent with a requirement for both motif i and ii of SRC-1 for PPAR γ activation. These studies suggest that appropriately spaced LXXLL motifs are essential for maximal SRC-1 function.

IV. X-RAY CRYSTAL STRUCTURES

Biochemical studies suggest that interactions between SRC coactivators and steroid/nuclear receptors involve LXXLL motifs of the coac-

tivators and the AF-2 helix of the receptors. Because steroid/nuclear receptors usually form dimers on DNA template, and SRC contains multiple LXXLL motifs, the mechanism of interaction is expected to be complex but precise to allow receptor specificity and coactivator selection. Recently, the interaction surface between SRC coactivator and receptor has been analyzed by X-ray crystallography studies and scanning mutagenesis studies. These studies led to the discovery of a hydrophobic cleft (groove) on the surface of receptor LBD, which appears to bind directly to the LXXLL motif of a given SRC coactivator. This hydrophobic cleft is induced upon agonist binding, consistent with ligand-dependent interaction. The interaction also involves the C terminus AF-2 helix (H12), which undergoes a drastic conformational change in response to ligand binding and forms part of the hydrophobic cleft. The interactions observed in the crystal structure are consistent with many biochemical data, and correlate precisely with the role of AF-2 helix in mediating both SRC interaction and ligand-dependent transcriptional activation (Kalkhoven *et al.*, 1998). This section summarizes the characteristics of the hydrophobic cleft and detailed mechanisms of the formation and composition of this coactivator-binding site.

A. HYDROPHOBIC CLEFT OF TR LBD

Based on the TR LBD X-ray crystallographic structure, 37 surface residues of hTR β 1 LBD were systemically mutated and tested for interactions with GRIP1 (Feng *et al.*, 1998). As expected, mutations of surface residues in helix 12 (L454R and E457K) of TR abolished GRIP1 binding. Two mutations in helix 3 (V284R and K288A) and two in helix 5 (I302R and K306A) also impaired binding, suggesting that both helix 3 and helix 5 also contribute to the formation of a coactivator-binding site. Point mutations that diminish GRIP1 binding (V284R, K288A, I302R, L454R, and E457K) also show decreased binding to SRC-1a, suggesting that different SRC coactivators may interact with a similar set of TR surface residues. Furthermore, transient transfection assay indicates that mutations with impaired GRIP1 binding also show diminished ligand-dependent transactivation function, which in turn could be partially restored by overexpression of GRIP1. Several control experiments demonstrate that these mutants are still efficient in hormone binding, heterodimerization, DNA binding, and inhibition of AP-1 activity, suggesting a direct involvement of coactivator binding in ligand-dependent transcriptional activation. The TR surface residues

required for binding to GRIP1 and SRC-1 are highly conserved among members of the steroid/nuclear receptor family, suggesting a similar coactivator-binding surface among different nuclear receptors. Consistently, the corresponding mutations (K362A, V376R, and E542K) in hER α also abolished GRIP1 binding and inhibited transcriptional activation. Similarly, the lysine 366 of mouse ER α , which aligns to the K362 residue in human ER α in the predicted helix 3, is also essential for E2-dependent transactivation and binding to coactivators SRC-1 and TIF2 (Henttu *et al.*, 1997).

The critical residues identified by the scanning surface mutagenesis for coactivator binding appear to encircle a small hydrophobic cleft on the surface of TR-LBD (Fig. 7). Ligand binding results in the formation

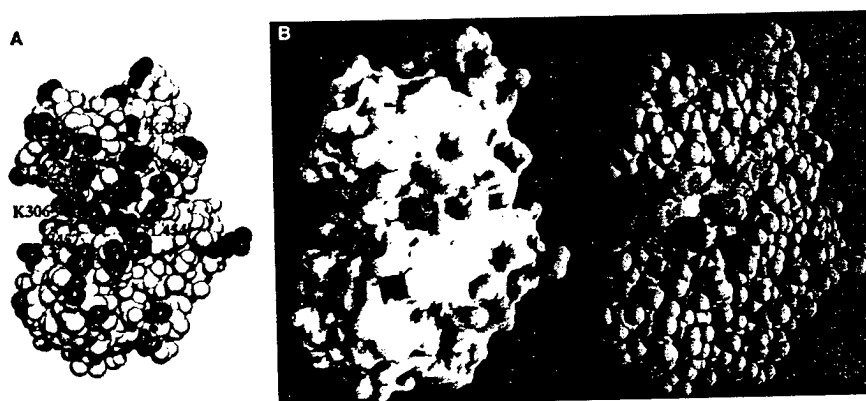


FIG. 7. A hydrophobic cleft on TR LBD involved in binding of SRC coactivators. A small cluster of effective mutations that surround a surface cleft containing central hydrophobic residues was identified by scanning surface mutagenesis. (A) A space-filling model of the TR LBD shows the LBD surface locations of mutations made in the full-length hTR β 1. Mutated residues that have no effect on GRIP1 binding or on activation in HeLa cells are shaded black. Mutated residues with diminished GRIP1 and SRC-1a binding and diminished activation in HeLa cells are shaded gray. (B) The AF-2 surface contains a cleft, one side of which is formed by conformationally hormone-responsive residues. Left, a view of the TR LBD molecular surface, showing the concave surfaces in gray; note the cavity at the center of the figure. Right, a space-filling model of the TR LBD, overlaid with a molecular surface view restricted to a 12-Å radius of the hydrophobic cavity. [Reprinted with permission from Feng W., *et al.* (1998). Hormone-dependent coactivator binding to a hydrophobic cleft on nuclear receptors. *Science* 280, 1747–1749. Copyright © 1998 American Association for the Advancement of Science.]

of this surface by folding the carboxyl-terminal AF-2 helix against a scaffold of H3, H4, and H5. It was predicted that this small hydrophobic cleft will match a complementary surface of the LXXLL motif with the hydrophobic residues driving coactivator-binding reaction (Feng *et al.*, 1998).

B. STRUCTURE OF TR β :LXXLL PEPTIDE COMPLEX

The interacting interface between TR LBD and the LXXLL motif has now been revealed by X-ray crystallography. The crystal structure of hTR β LBD complexed with T3 (3,3',5-triiodo-L-thyronine) and a 13-aa peptide KHKILHTLLQDSS encompassing the LXXLL motif ii of GRIP1 was determined (Darimont *et al.*, 1998). The crystal contains two asymmetric monomers of the TR β LBD with each monomer binding to one peptide. The structure of the hTR β LBD is similar to that of the rTR α LBD (Wagner *et al.*, 1995) and consists of 12 α -helices and 4 β -strands organized in three layers. The LXXLL peptide forms an amphipathic α -helix of about three turns for the core residues. The helical structure of the peptide may be induced by complex formation since far UV-CD spectrum of the peptide indicates a random coil conformation in the absence of TR β LBD. In the crystal structure, the hydrophobic face of the peptide helix contacts a hydrophobic groove formed by 16 residues from helices H3, H4, H5, and H12 of the hTR β LBD. The 16 residues are I280, T281, V283, V284, A287, and K288 from H3; F293 from H4; Q301, I302, L305, K306, and C308 from H5; and L454, E457, V458, and F459 from H12. These residues are arranged in a way that the hydrophobic residues form the floor of the groove and the charged residues line the rim. The three leucines of the LXXLL core, L690, L693, and L694, are buried within the hydrophobic groove (Fig. 8). The L690 residue makes van der Waals contacts with L454 and V458 of H12, and I689 packs against L454 of H12 outside the edge of the groove. L693 contacts V284 of H3, whereas L694 contacts F293 and L305 of H4 and H5, respectively. This structure is consistent with results obtained in scanning surface mutagenesis, confirming the importance of V284 of H3 and L454 of H12 for *in vitro* binding with both GRIP1 and SRC-1a (Feng *et al.*, 1998).

C. STRUCTURE OF THE ER α :LXXLL PEPTIDE COMPLEX

The crystal structure of diethylstilbestrol (DES)-bound ER α LBD complexed with a LXXLL peptide (motif ii) of GRIP1 has also been determined (Shiau *et al.*, 1998). The overall structure of the ER-peptide

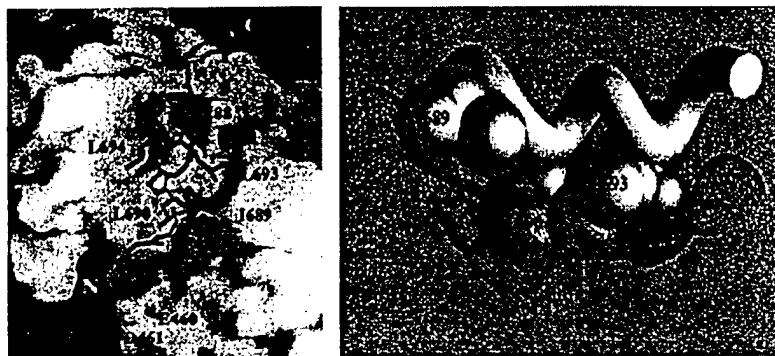


FIG. 8. The TR β LBD:LXXLL peptide interface revealed by X-ray crystal structure. The side chains of the LXXLL motif ii of GRIP1 are shown in a CPK representation, with the main chain of the peptide drawn as a C α worm. The three leucines fit into pockets on the molecular surface of the TR β LBD, depicted as mesh, whereas the nonconserved isoleucine residue rests on the edge of the surface cleft. [Adapted by permission from Fig. 4 of Darimont, B. D., *et al.* (1998). Structure and specificity of nuclear receptor coactivator interactions. *Genes & Development* 12, 3343–3356.]

complex is similar to that of the TR-peptide complex (Darimont *et al.*, 1998). The LXXLL peptide binds as a short amphipathic α -helix to a hydrophobic groove formed on the surface of the LBD (Fig. 9). This LXXLL binding surface of ER LBD is located at the same position as the hydrophobic cleft of TR LBD. The asymmetric unit of the ER complex contains a noncrystallographic dimer (Tanenbaum *et al.*, 1998; Brzozowski *et al.*, 1997; Shiau *et al.*, 1998), consistent with ER's function as a homodimer (Mangelsdorf and Evans, 1995). In the ternary complex, one LXXLL peptide is bound to each LBD in a hydrophobic groove composed of residues from helices H3, H4, H5, and H12 and the turn between H3 and H4 (Fig. 9A). The ends of this hydrophobic groove are charged, similar to the coactivator-binding pockets found in the TR-LXXLL peptide complex (Darimont *et al.*, 1998) (Fig. 9C). In the crystal structure, L690 forms van der Waals contacts with I358, V376, L379, E380, and M543, whereas L694 makes van der Waals contacts with I358, K362, L372, Q375, V376, and L379 of the ER LBD on the hydrophobic floor. In contrast, I689 and L693 of the LXXLL helix rest against the rim of the groove. The side chain of I689 lies in a depression formed by D538, L539, and E542 and the side chain of L693 makes nonpolar contacts with I358 and L539 of the ER LBD. In addition to the hydrophobic interactions, the LXXLL helix appears to be stabilized by capping interactions with E542 and K362 of ER LBD at opposite ends of the LXXLL

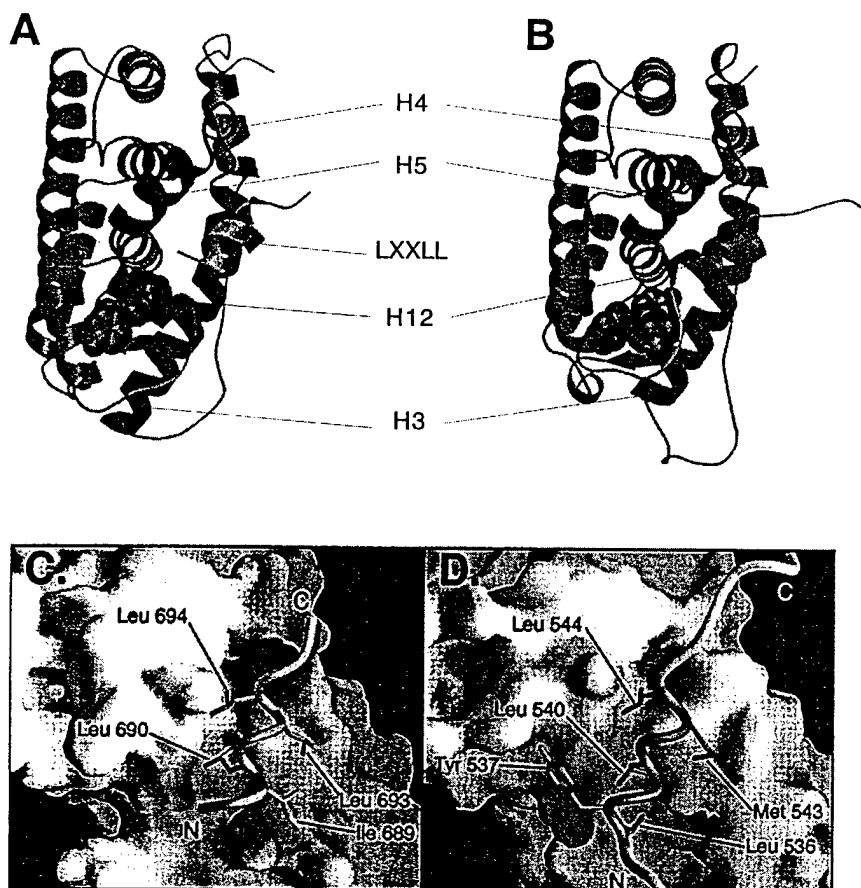


FIG. 9. Structure of ER α -LXXLL Peptide Complex. (A) Structure of the DES-ER-peptide complex. The coactivator peptide and the LBD are shown as ribbon drawings. DES is shown in space-filling representation. (B) Structure of the OHT-ER LBD complex. The LBD is depicted as a ribbon drawing. OHT is shown in space-filling representation. (C) A molecular surface representation of the LBD bound to DES. The side chains of Leu-690 and Leu-694 of the coactivator peptide are bound in a hydrophobic groove and those of Ile-689 and Leu-693 rest against the edge of this groove. (D) A molecular surface representation of the LBD bound to OHT. Whereas the side chains of Leu-540 and Leu-544 of helix 12 are embedded in the hydrophobic groove, that of Met-543 lies along the edge of this groove. [Adapted by permission from Figs. 2 and 3 of Shiau, A. K., *et al.* (1998). The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* 95, 927-937.]

helix by forming hydrogen bonds. The importance of these interactions observed in the crystal structures was confirmed by a series of site-directed mutations of the ER LBD. Mutations that perturb the hydrophobic characteristic of the LXXLL-binding groove, or that prevent the formation of the capping interactions (K362A and E542K), abolish ligand-dependent interaction between ER and GRIP1 (Shiau *et al.*, 1998), indicating that both capping and hydrophobic packing interactions are important.

Importantly, the coactivator-binding hydrophobic groove of ER LBD is occluded in the LBD bound with the antagonists 4-hydroxytamoxifen (OHT) (Shiau *et al.*, 1998) or Raloxifen (RAL) (Brzozowski *et al.*, 1997) due to misalignment of the AF-2 helix (Fig. 9B). This AF-2 helix appears to mimic the interactions of the LXXLL peptide with the LBD in the antagonist-bound complex, providing a molecular basis for mechanism of antagonism by OHT and RAL. In the crystal structures, the ER agonists DES and E2 are completely buried within a hydrophobic cavity of the LBDs (Brzozowski *et al.*, 1997; Shiau *et al.*, 1998). In contrast, the binding of antagonist OHT or RAL induces a conformation that differs from the structure driven by DES or E2 binding. Thus, the receptor antagonism by OHT and RAL is attributed to their bulky side chains that project out of the ligand-binding pocket between helices 3 and 11. Consequently, the positive-charged side chains of OHT and RAL produce steric clashes with the hydrophobic side chain of L540 in AF-2 helix, shifting this helix from over the ligand-binding pocket to the hydrophobic region of the LXXLL-binding groove (Fig. 9B). It appears that an LXXLL-like sequence (LXXML) within ER helix 12 binds intramolecularly to the coactivator-binding pocket of LBD (Fig. 9D). Therefore, the binding of antagonist to ER promotes an AF-2 helix conformation that cripples the AF-2 surface and inhibits binding of SRC coactivator by blocking the hydrophobic groove required for binding of LXXLL motif. Since the LXXLL-like motif of ER is not shared by all other nuclear receptors, other mechanisms of antagonism might be utilized by different steroid/nuclear receptors.

D. STRUCTURE OF THE PPAR γ :SRC-1 COMPLEX

The crystal structures of an apo-PPAR γ LBD and a ternary complex containing the PPAR γ LBD, the antidiabetic ligand rosiglitazone (BRL49653), and an 88-aa fragment of SRC-1 have also been described recently (Nolte *et al.*, 1998) (Fig. 10). In both the apo and the ternary complex structures, the PPAR γ LBD forms a noncrystallographic dimer, similar to the RXR α and ER α crystal structures (Bourguet *et al.*, 1995;

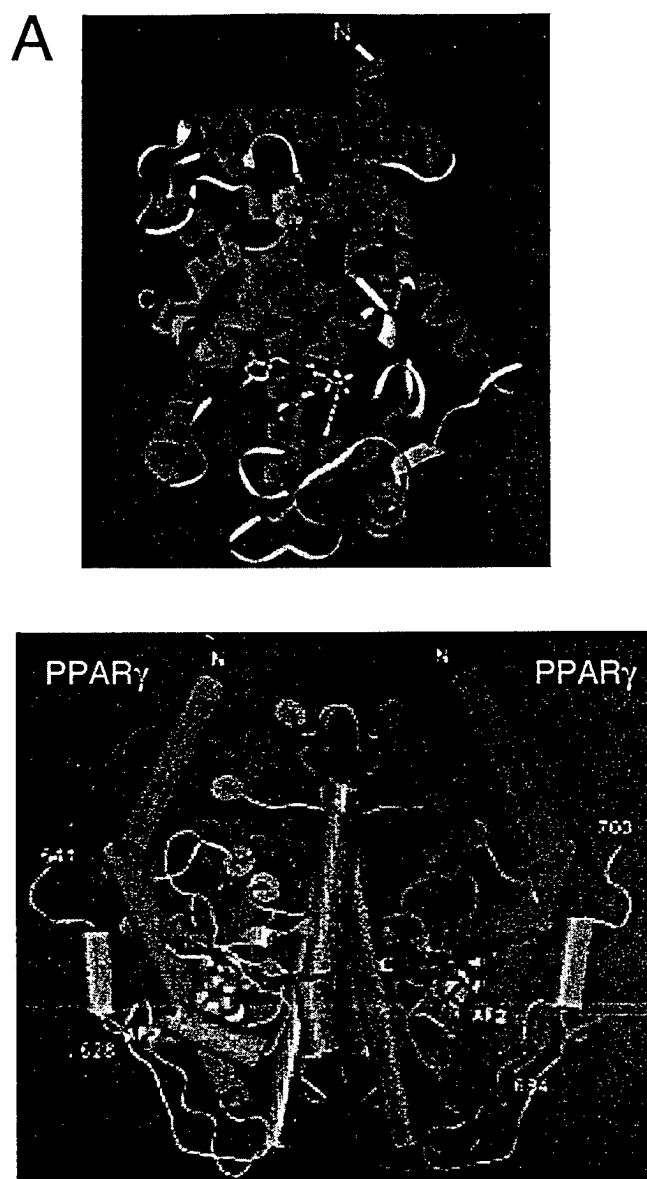


FIG. 10. Structure of the PPAR γ -rosiglitazone-SRC-1 ternary complex. (A) Ribbon drawing showing the ternary complex of PPAR γ LBD, BRL49653, and the LXXLL helix domain of SRC-1. Rosiglitazone (stick diagram) binds in a deep cavity of the protein and provides a network of polar interactions that include the AF-2 domain. (B) Ribbon drawing of the PPAR γ LBD dimer and SRC-1, including the ligand rosiglitazone. The struc-

Brzozowski *et al.*, 1997). The structure of the PPAR γ LBD is very similar to the overall fold of other steroid/nuclear receptors, except that it contains an extra helix between the first β -strand and H3. The PPAR γ structure reveals a large T-shaped ligand-binding pocket. The ligand rosiglitazone occupies about 40% of this cavity in the ternary complex. The remaining cavity of the ligand-binding pocket may allow free interaction with ligands in a relatively nonspecific manner, resulting in flexibility on ligand binding by PPAR γ .

The crystal structure of the PPAR γ -rosiglitazone-SRC-1 ternary complex shows that SRC-1 binds to a liganded PPAR γ homodimer, with one LXXLL binding to one molecule and the second LXXLL binding to the other molecule (Fig. 10B). The connecting sequences between these two LXXLL motifs of SRC-1 were not defined. In the ternary complex, E471 and K301 of PPAR γ appear to define a "charge clamp" that allows the placement of LXXLL motif into the coactivator-binding site (Fig. 10A). At one end of the coactivator-binding site, the side chain of E471 forms hydrogen bonds with the backbone amides of K632 and L633 in motif i and with the backbone amides of K688, I689, and L690 in motif ii. At the other end of the binding site, the side chain of K301 forms hydrogen bonds with two backbone carbonyls of L636 and T639 in motif i and L693 and L694 in motif ii. The corresponding residues of both E471 and K301 in TR and ER also are important in coactivator binding and transactivation (Henttu *et al.*, 1997; Darimont *et al.*, 1998). Similar to the TR-LXXLL and ER-LXXLL complexes, the hydrophobic face of the LXXLL helix of SRC-1, packs into a hydrophobic pocket formed between E471 and K301 by H3, H4, H5, and H12 of PPAR γ . The hydrophobic core of the LXXLL motif is buried within the binding surface and amino acids L633, L636 and L690, L693 of the two SRC-1 LXXLL motifs interact hydrophobically with L468 and L318 of the PPAR γ LBD. The residues at positions -3 and -2 of the LXXLL motif do not appear to make any significant interactions with the LBD. The amino acid at position -1 fits in a shallow pocket created by P467 and L468 of the AF-2 helix H12 and the +4 residue of the LXXLL motif. The amino acids

ture of SRC-1 was determined from amino acids 628-640 and 684-703 and was crystallographically refined. Very weak electron density from residues 670 to 684 was visible but was not crystallographically refined and is shown as a dashed line. SRC-1 amino acids 642-669 were disordered and not structurally determined. The diagram shows how one SRC-1 molecule, with two interacting domains, forms a complex with a PPAR γ homodimer. The dashed line connecting the two structurally determined domains of SRC-1 is the proposed connection between these two domains. [Adapted by permission from Figs. 2 and 3 of Nolte, R. T., *et al.* (1998). Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor- γ . *Nature* 395, 137-143.]

at +2 and +3 of the LXXLL motif face out into solution and make no contacts with the LBD, consistent with the lack of sequence conservation and other mutagenesis studies (McInerney *et al.*, 1998; Darimont *et al.*, 1998). The two leucines at positions +4 and +5 lie in a hydrophobic pocket and, therefore, are most critical for stabilizing the interaction by forming hydrogen bonds with the clamping residue K301 of the LBD.

According to this model, the length and orientation of the LXXLL motif is vital for proper backbone interactions with E471 in AF-2 helix and with K301 in helix H3. The E471 and K301 are highly conserved among nuclear receptors and are important for transcriptional activation and coactivator binding. These two residues appear to define a ligand-dependent "charge clamp" that positions the LXXLL motif into a hydrophobic pocket in the receptor LBD. The observation that two LXXLL motifs of one SRC-1 molecule make simultaneous contact with a PPAR γ homodimer suggests a cooperative binding of the LXXLL motifs to a receptor dimer. The existence of a third LXXLL motif within SRC coactivators may allow combinatorial regulation and optimal interaction for different receptors. In the active ternary complex, the two PPAR γ LBDs have nearly identical conformations. In contrast, one AF-2 helix in the apo-PPAR γ homodimer adopts an extended inactive conformation, projecting away from the LBD, whereas the other AF-2 helix is folded against the LBD, adopting an active conformation. It is possible that the unliganded receptor can assume both active and inactive conformations, with the ligand acting to lock the receptor into the active conformation as proposed by the "mouse trap" model (Renaud *et al.*, 1995). However, the "inactive" AF-2 helix appears to contact the charge clamp of the active AF-2 helix in a crystallographically related PPAR γ molecule. It is believed that this arrangement of an AF-2 helix in the LXXLL binding pocket may underlie allosteric inhibition observed with specific partners of RXR.

E. MODEL OF ALLOSTERIC INHIBITION

Functional studies suggested that RXR-PPAR heterodimer could be activated by both PPAR and RXR ligands, whereas RXR-RAR heterodimer is selectively activated by RAR ligand only (Kliwer *et al.*, 1992; Kurokawa *et al.*, 1994). The differential ligand responsiveness may be due to allosteric inhibition of the binding of ligands to RXR by RAR, but not by PPAR, in the respective heterodimers. It was proposed that allosteric inhibition of RXR by RAR is a result of the placement of

RXR AF-2 helix in the LXXLL-binding pocket of RAR in the absence of ligand (Westin *et al.*, 1998). Upon binding of RAR ligands, an LXXLL motif is recruited, displacing the RXR AF-2 helix and allowing RXR ligands to bind (Fig. 11). Consequently, the second LXXLL motif will then bind to the RXR molecule.

This model is supported by several observations. First, an RXR-specific ligand LG268 can only stimulate the binding of SRC-1 to RXR-RAR heterodimers in the presence of an RAR-specific ligand TTNPB, indicating that the interaction of SRC-1 with RAR may relieve the

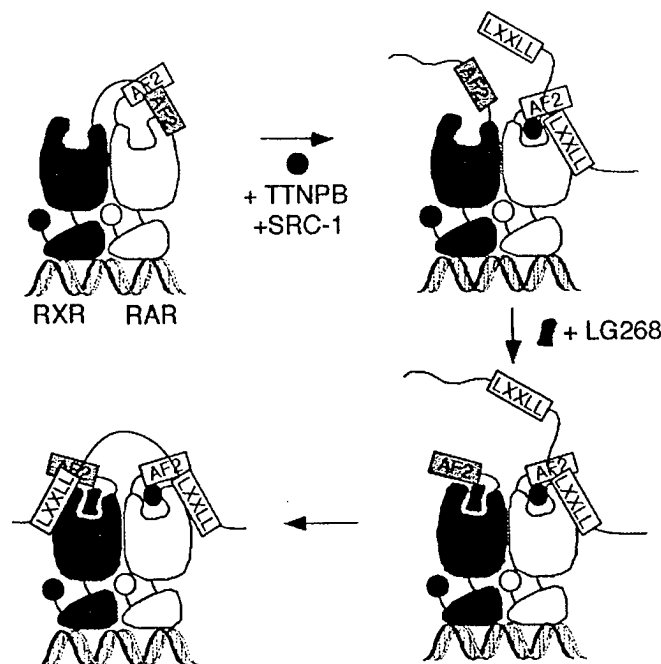


FIG. 11. Model of allosteric inhibition of RXR-RAR heterodimer. In the absence of ligand, the AF-2 helix of RXR is docked to the RAR coactivator-interaction site, preventing the binding of RXR ligands. In response to RAR-specific ligand, one of the three LXXLL motifs is recruited to RAR, resulting in displacement of the RXR AF-2 helix from RAR (step 1). The release of the RXR AF-2 domain relieves allosteric inhibition, allowing ligands to bind to RXR (step 2). The binding of an RXR ligand can then promote the interaction of a second LXXLL motif from the same SRC-1 molecule with RXR, stabilizing the complex (step 3). [Adapted by permission from Fig. 5 of Westin, S., *et al.* (1998). Interaction controlling the assembly of nuclear-receptor heterodimers and co-activators. *Nature* 395, 199–202.]

allosteric inhibition on RXR. Accordingly, binding of the RXR-specific ligand LG69 to RXR-RAR heterodimers was induced by combination of TTNPB with SRC-1, suggesting that activation of RAR by TTNPB and SRC-1 permits binding of ligands to RXR. Consistent with this, overexpression of SRC-1 also enhances transcription induced by RXR-specific ligand LG268 in the presence of TTNPB. These studies suggest that ligand activation of RAR recruits coactivators, which may relieve allosteric inhibition on RXR, allowing RXR to bind ligands and to interact with coactivators. Second, two LXXLL motifs of SRC-1 are required for the cooperative effects of two ligands on binding of SRC-1 to a heterodimer of RXR-RAR or PPAR γ -RXR. This suggests that each LXXLL motif may contact one molecule of the dimer. Third, both AF-2 domains of the RXR-RAR heterodimer are required for the cooperative effects of two ligands to recruit SRC-1. Deletion of an AF-2 helix from one receptor partially increases SRC-1 binding to the partner and completely blocks the cooperative effects of two ligands to recruit SRC-1. These data suggest an inhibitory role of the AF-2 helix on SRC-1 binding to the partner and that both AF-2 domains of the heterodimer are required for cooperative recruitment of SRC-1. Fourth, the X-ray crystal structure of apo-PPAR γ reveals that the AF-2 helix of one PPAR γ molecule interacts with the LXXLL binding pocket of another PPAR γ in a different, crystallographically related dimer. Molecular modeling of the RXR-RAR heterodimer shows that the AF-2 helix of RXR could be rotated to contact the LXXLL-binding pocket of RAR. Presumably, such an interaction would prevent AF-2 helix-dependent closure of the ligand-binding pocket of RXR, suggesting a structural basis for allosteric inhibition by RAR on ligand binding of RXR. Also, the RXR AF-2 helix is required for binding of RXR ligands since RXR Δ 443-RAR heterodimer does not bind well to RXR-specific ligand in the presence of TTNPB and SRC-1. In addition, synthetic coactivator LXXLL peptides can relieve the inhibition on RXR ligand binding. A synthetic RXR AF-2 peptide binds to the unliganded RAR with a higher affinity than the coactivator LXXLL peptide, and binding of RXR AF-2 peptide to RAR is displaced from RAR by LXXLL peptides. Finally, GST-RXR-AF2 helix fusion protein binds to RAR efficiently, and such binding is inhibited by SRC-1 in the presence of RAR ligand. In contrast, GST-RXR-AF2-helix interacts poorly with PPAR γ , consistent with the observation that PPAR γ does not inhibit ligand binding of RXR.

Consistent with the model, mutations in the AF-2 helix of ER that affect its AF-2 function and mutations that affect dimerization both impair SRC-1 binding (Kalkhoven *et al.*, 1998). For instance, R507A and L511A mutations in ER that do not affect hormone binding appear to

inhibit binding of SRC-1 (Kalkhoven *et al.*, 1998). Conversely, the G525R mutation, which still allows dimerization but is unable to bind ligands, also inhibits binding of SRC-1. In addition, while SRC-1 interacts with an ER homodimer containing two functional AF-2 domains in a gel retardation assay, SRC-1 could not form a complex with an ER homodimer containing defective AF-2 helix (Kalkhoven *et al.*, 1998). These studies suggest that, in addition to hormone binding and AF-2 function, homodimerization of ER is also required for efficient recruitment of SRC-1. Together, these studies support a hypothesis that one coactivator molecule interacts simultaneously with two subunits of the receptor homo- or heterodimer through two LXXLL motifs of one coactivator molecule. However, another study using gel shift assay for analyzing interaction between rTIF2 and TR/RXR heterodimer concluded that two coactivator molecules bind to a heterodimeric receptor complex (Leers *et al.*, 1998). Further studies are necessary to understand the exact stoichiometry of the coactivator-receptor complex and the possible differences among different receptor-coactivator complexes.

V. MECHANISM OF TRANSACTIVATION

A. ACTIVATION DOMAINS

Modulation of the transcriptional activities of steroid/nuclear receptors by coactivators is a complex process involving enzymatic remodeling of chromatin as well as communication with basal transcriptional machinery at specific promoters. One common property of transcriptional coactivators is the ability to activate transcription when recruited to a promoter via protein-protein interaction with DNA binding proteins. Such a recruitment event can be mimicked by fusing coactivator with a heterologous DBD. Using Gal4-DBD fusion, all three SRC coactivators have been shown to contain intrinsic transcription activation function (Li *et al.*, 1997; Zhu *et al.*, 1996; Torchia *et al.*, 1997; Voegel *et al.*, 1998; Suen *et al.*, 1998; Hong *et al.*, 1997; Chen *et al.*, 1997; Kurokawa *et al.*, 1998; Oñate *et al.*, 1998) (Fig. 12). Fusion proteins of Gal4-DBD and full-length mSRC-1 (Zhu *et al.*, 1996), GRIP1 (Hong *et al.*, 1997), NCoA-1 and p/CIP (Torchia *et al.*, 1997) efficiently activate transcription from a Gal4-driven promoter in both mammalian and yeast cells. Comparison of the transactivation activity between Gal4-NCoA1 and Gal4-p/CIP suggests stronger activation function for NCoA-1 than p/CIP (Torchia *et al.*, 1997). This is consistent with a finding in the same study that p/CIP exhibits three- to fivefold less coacti-

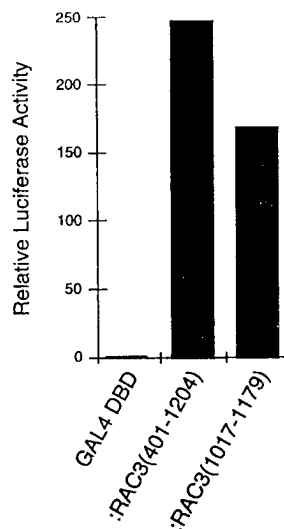


FIG. 12. Transcriptional activation by the SRC coactivator. Transcriptional activation by RAC3 in mammalian cells. The indicated RAC3 fragments were expressed as Gal4-DBD fusion proteins from the CMV promoter. The relative fold-induction is determined by comparing with activity of Gal4-DBD.

vation activity on RAR and ER than NCoA-1. However, all three SRC coactivators contain potent transcriptional activation domains, and SRC-3 and SRC-1 have comparable levels of coactivation function on RAR, PR, and TR (Li *et al.*, 1997; Takeshita *et al.*, 1997).

Within SRC-1, three independent activation domains have been described. One study found the first activation domain (AD1) at the N-terminal 93 amino acids that cover the bHLH region (Oñate *et al.*, 1998). However, another study showed no detectable transactivation function with a fusion of Gal4 DBD and amino acids 1–198 of hSRC-1 (Kalkhoven *et al.*, 1998). Therefore, a transcriptional suppressor domain may exist within the conserved PAS-A region. The second activation domain (AD2) was mapped to amino acids 781–988 or 840–948 of hSRC1 (Oñate *et al.*, 1998; Kalkhoven *et al.*, 1998), and 896–1200 or 947–1084 of NCoA-1 (mSRC-1) (McInerney *et al.*, 1998; Kurokawa *et al.*, 1998). The AD2 domains in SRC-2 and SRC-3 have been mapped to amino acids 1010–1131 of TIF2 (Voegel *et al.*, 1998), 1017–1179 of RAC3 (Li *et al.*, 1997), 1038–1088 of ACTR (Chen *et al.*, 1997), and 896–1200 of p/CIP (Kurokawa *et al.*, 1998). Therefore, the minimal AD2 of SRC-3 is located within a 50-aa fragment. Direct comparison of the AD2 activity between p/CIP and NCoA-1 shows that they can activate transcrip-

tion equally well (Kurokawa *et al.*, 1998). In addition to AD1 and AD2 domains, a third activation domain (AD3) was observed in all three SRC coactivators. The AD3 domain has been mapped to amino acid residues 1241–1385 of SRC-1a (Kalkhoven *et al.*, 1998). In contrast, little activation was observed with amino acid 948–1441 or 1241–1441 fragment of SRC-1a (Oñate *et al.*, 1998; Kalkhoven *et al.*, 1998). Therefore, the C-terminal 56 amino acids may inhibit transactivation of AD3 (Kalkhoven *et al.*, 1998). This putative C-terminal suppressor domain contains a LXXLL motif that can interact with liganded receptors; it is conceivable that protein–protein interaction of this LXXLL motif with the receptors might regulate AD3 activity. It is currently unclear whether a similar suppressor domain exists in SRC-2 or SRC-3. However, both SRC-2 and SRC-3 lack a C-terminal LXXLL motif, suggesting that the mechanism of transcriptional activation may differ among members of the SRC coactivator family.

B. INTERACTION WITH CBP/p300

The ability of SRC coactivator to activate transcription has been linked, at least in part, to interaction with CBP/p300. The interaction between SRC and CBP/p300 was first revealed by identification of SRC-1 as a CBP/p300 binding protein (Yao *et al.*, 1996; Kamei *et al.*, 1996). A mouse cDNA fragment encoding amino acid residues 789–993 of SRC-1 was isolated in a search for p300-binding proteins in a yeast two-hybrid screen (Yao *et al.*, 1996). The association of mSRC-1 with p300 has been confirmed by GST pull-down, coimmunoprecipitation, and subcellular colocalization assays, and the interaction surface on p300 was mapped to the C-terminal 308 amino acids (Yao *et al.*, 1996). In addition, mSRC-1 was also isolated as a CBP interacting protein in a far-Western-based screening (Kamei *et al.*, 1996). In addition to interaction with nuclear receptors through the N-terminal region of CBP/p300, the C-terminal fragment between amino acids 2058–2163 of CBP appears to interact with SRC coactivators. Similarly, SRC-2 and SRC-3 have also been shown to interact with CBP/p300 (Torchia *et al.*, 1997; Voegel *et al.*, 1998; Li and Chen, 1997). The CBP/p300 interacting domain of TIF2 was mapped to amino acids 1010–1131 by GST pull-down assay (Voegel *et al.*, 1998). Similarly, the CBP interacting domain of RAC3 was mapped to amino acids 1017–1179 by far-Western analysis (Li and Chen, 1997) (Fig. 13), or to amino acids 947–1084 of p/CIP in yeast two-hybrid assay (Torchia *et al.*, 1997).

The CBP/p300 interacting domain of the SRC coactivator contains three conserved regions similar to the LXXLL motifs involved in re-

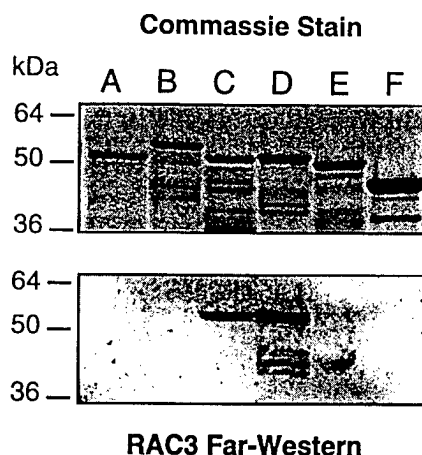


FIG. 13. SRC coactivator interacts with CBP. Coomassie blue staining of the purified GST-CBP fusion proteins (top). The interaction of full-length RAC3 with GST-CBP fragments was probed in a far-Western blot. The A to F fragments of CBP contain amino acid residues 1678–1880, 1801–2000, 1921–2120, 2041–2240, 2161–2360, and 2301–2441, respectively.

ceptor interaction (Fig. 5). The role of these LXXLL motifs in mediating the interaction with CBP/p300 has been analyzed (Voegel *et al.*, 1998; McInerney *et al.*, 1998). In one study, individual deletion of motif iv, motif v, or motif vi of TIF2 has no significant effect on CBP interaction (Voegel *et al.*, 1998). However, mutation of three leucines in the LLXXL core of motif v to alanines, but not alteration of the middle XX residues to alanines, significantly reduces CBP interaction with TIF2 (Voegel *et al.*, 1998), suggesting that motif v is important for CBP interaction. In contrast, mutation of motif iv of NCoA-1 from LXXLL to LAAAA impairs NCoA-1's capability to rescue IgG-inhibited RAR transactivation, while mutation of motif v had no effect (McInerney *et al.*, 1998). Double mutation of motif iv and motif v completely blocks the ability of NCoA-1 to rescue transactivation by RAR, TR, and PPAR γ . The motif iv and v double mutant also fails to interact with CBP (McInerney *et al.*, 1998). These data suggest that the conserved LXXLL motifs within the CBP interacting domain of SRC coactivators play an important role in mediating the interaction. It appears that motif iv is more important for SRC-1, while motif v is most critical for TIF2 in CBP binding. Conversely, multiple helices within the SRC interacting domain of CBP are required to various degrees for interaction with NCoA-

1 (McInerney *et al.*, 1998). A single-point mutation K2109A in CBP significantly impairs its interaction with NCoA-1. The predicted structure of the SRC-interacting domain on CBP suggests a hydrophobic binding pocket, analogous to the nuclear receptor-binding pocket, by which the LXXLL motifs of SRC bind to CBP/p300. Because interactions between CREB and the KIX domains of CBP are dependent on phosphorylation, analogous induced-fit events may also regulate the interaction between SRC and CBP/p300.

Comparison of the CBP/p300-interacting domain and the AD2 domain of the SRC coactivator indicates that these two domains overlap with one another (Li and Chen, 1997; McInerney *et al.*, 1996; Voegel *et al.*, 1998). Mutations of TIF2 that affect CBP interaction also inhibit transcriptional activation (Voegel *et al.*, 1998). By analyses of 13 deletion mutants and 2 point mutants generated within residues 1011–1122 of TIF2, all mutants that retain the ability to interact with CBP also activate transcription. In particular, point mutation within motif v of TIF2 that replaces the three leucines with alanines affects both CBP interaction and transcriptional activation. Accordingly, the TIF2 (LLL) mutant showed diminished ER coactivation function (Voegel *et al.*, 1998). These studies suggest that interaction with CBP may underscore the ability of TIF2 to activate transcription.

The requirement of CBP for transcriptional activation by SRC-1 (NCoA-1) and for enhancing transcription by nuclear receptors has also been analyzed by microinjection assay (McInerney *et al.*, 1998). Mutation of the two LXXLL motifs (iv and v) within NCoA1 appears to abolish the function of NCoA-1 in both CBP interaction and coactivation for RAR, TR, and PPAR γ . Furthermore, injection of anti-CBP IgG also abolishes transcriptional activation by NCoA-1. Therefore, CBP/p300 interaction is essential for transcriptional activation and coactivation function of SRC-1. Accordingly, microinjection of anti-CBP IgG inhibits RA-dependent transactivation, indicating that CBP is required for RAR-mediated transactivation. Because the N terminus of CBP/p300 also interacts with nuclear receptors, the relative contribution of nuclear receptor interacting domain and the SRC interacting domain on RAR transactivation was tested by antibody microinjection and rescue experiment. It appears that the nuclear receptor interacting domain of CBP is not required to stimulate RAR transactivation. In contrast, the SRC interacting domain is essential for stimulating RAR transactivation (McInerney *et al.*, 1998). These results are in agreement with a bridging hypothesis that SRC coactivators function by recruiting CBP/p300 coactivators to specific promoters.

C. INTERACTION WITH P/CAF

In addition to CBP/p300, SRC-1 and ACTR have also been shown to interact with P/CAF, a p300/CBP-associated histone acetyltransferase (Spencer *et al.*, 1997; Chen *et al.*, 1997). P/CAF appears to interact with SRC-1 fragment spanning residues 1027–1139 and 1139–1250, suggesting two independent interaction regions for P/CAF (Spencer *et al.*, 1997). A Gal4-P/CAF fusion protein also interacts with SRC-1 fragments 360–1139, 1138–1441, and 1216–1441 in a mammalian two-hybrid assay. Similar to SRC-1, ACTR was also shown to interact with P/CAF (Chen *et al.*, 1997). The interaction between P/CAF and ACTR was shown by both GST pull-down and yeast two-hybrid assays (Chen *et al.*, 1997). The ability of both SRC-1 and ACTR to interact independently with CBP/p300 and P/CAF provides a molecular scaffold to bridge the HAT protein complex to DNA-bound steroid/nuclear receptors. However, the interaction of SRC coactivators with P/CAF does not correlate with the transcriptional activity of SRC coactivators since the transcriptional activation domain and P/CAF interacting region are separable (Chen *et al.*, 1997). In addition, fusion of P/CAF with Gal4-DBD is unable to activate transcription, suggesting that histone acetylation alone is not sufficient for transcriptional activation by SRC coactivators or P/CAF.

D. HISTONE ACETYLATION BY SRC COACTIVATORS

Transcriptional coactivators are thought to stimulate transcription by facilitating the assembly of active basal transcriptional machinery. How SRC coactivators gain access to repressed chromatin remains largely unknown. Transcriptionally active chromatin usually contains hyperacetylated histones (Brownell and Allis, 1996; Wade *et al.*, 1997). Accordingly, several transcriptional coactivators including the general coactivators CBP/p300 and its associated protein P/CAF are potent histone acetyltransferases (Yang *et al.*, 1996; Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996). Interestingly, both SRC-1 and ACTR also exhibit moderate intrinsic histone acetyltransferase activity (Spencer *et al.*, 1997; Chen *et al.*, 1997) and are capable of acetylating free and mononucleosomal histones with substrate preference on histones H3 and H4.

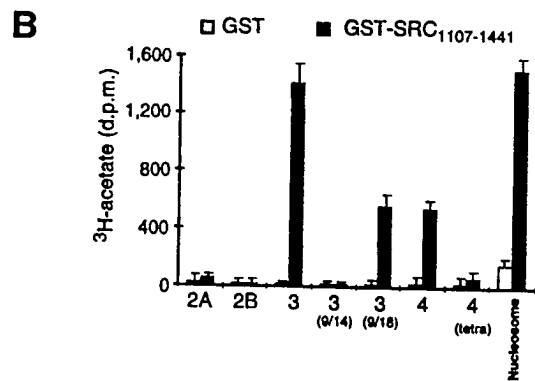
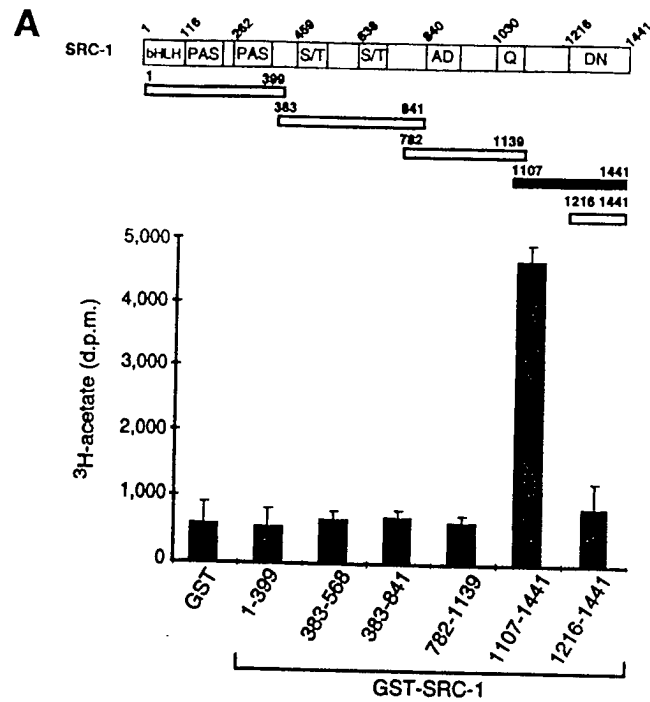
Histone acetylation by SRC-1 was first demonstrated in a filter-binding assay using SRC-1 immunoprecipitates obtained from COS cell extract (IP-HAT) (Spencer *et al.*, 1997) (Fig. 14A). The intrinsic HAT activity of SRC-1 was confirmed by an active gel assay in which

the immunoprecipitate was resolved by SDS-PAGE, and the HAT activity of individual polypeptide was determined. Intrinsic HAT activity was also detected with ACTR expressed in a baculovirus system (Chen *et al.*, 1997). The HAT activity of ACTR was confirmed by resolving the purified ACTR on a glycerol gradient and analyzing the HAT activity of each fraction, which revealed cofractionation of HAT activity and ACTR protein. It remains unclear whether SRC-2 (GRIP1/TIF2) also contains HAT activity or interacts with P/CAF (Voegel *et al.*, 1998).

The HAT domains were mapped to the C-terminal fragments at 1107–1441 and 1029–1292 of hSRC-1 and ACTR, respectively (Chen *et al.*, 1997; Spencer *et al.*, 1997). The HAT domain of ACTR lies between two activation domains, indicating that the HAT activity is not directly responsible for transcriptional activation by ACTR. Therefore, histone acetylation by SRC-1, ACTR, and P/CAF appears to be insufficient for transcriptional activation. Transcriptional activation by liganded nuclear receptors may involve a highly coordinated multistep action that could be facilitated by the SRC coactivators. Unlike CBP, which acetylates H2A and H2B in addition to H3 and H4 (Ogryzko *et al.*, 1996; Bannister and Kouzarides, 1996), both SRC-1 and ACTR acetylate only H3 and H4 with a preference for H3 (Chen *et al.*, 1997; Spencer *et al.*, 1997). This acetylation occurs on both free form and mononucleosomal histones, and packing of histones into nucleosome severely reduces the efficacy of acetylation. In addition, the acetylation sites have been identified using synthetic peptides corresponding to the N-terminal tails of H3 and H4 (Spencer *et al.*, 1997). The H3 peptide with preacetylated lysines 9 and 18 remains a good substrate, but preacetylation of lysines 9 and 14 inhibits SRC-1-mediated acetylation. Thus, lysine 14 and perhaps lysine 9 of histone H3 are the preferred sites for acetylation by SRC-1 (Fig. 14B). It is currently unknown whether ACTR acetylates distinct sites on histones, and if acetylation by SRC-1 and ACTR has different functional consequences.

E. INTERACTION WITH CYCLIN D1

Cyclin D1 forms complexes with CDKs in response to mitogenic stimulation and regulates cell cycle progression through the G₁ phase. Importantly, cyclin D1 is amplified and overexpressed in several human malignancies. Elevated levels of cyclin D1 are observed in up to 50% of human breast cancers (Donnellan and Chetty, 1998; Beijersbergen and Bernards, 1996). Cyclin D1 has been shown to interact and enhance ER-mediated transactivation in a ligand- and CDK-independent manner



| | | | |
|---------|----------------------|--------|-----------------------|
| H2A | SGGKGGKAGSAAKASQSRSA | H3 | ARTKQTARKSTGGKAPRKQL |
| H2B | SAKAEKKPASKAPAEKKPAA | H3 | ARTKQTARKSTGGKAPRKQLC |
| | | (9/14) | Ac Ac |
| H4 | SGRGKGGKGLGKGGAKRHRK | H3 | ARTKQTARKSTGGKAPRKQL |
| | | (9/18) | Ac Ac |
| H4 | SGRGKGGKGLGKGGAKRHRK | | |
| (tetra) | Ac Ac Ac Ac | | |

(Neuman *et al.*, 1997; Zwijsen *et al.*, 1997). Recently, cyclin D1 was shown to recruit SRC-1 to ER in the absence of ligand (Zwijsen *et al.*, 1998), accounting at least partly for ligand-independent activation of ER. Cyclin D1 mutant that fails to interact with SRC-1 inhibits cyclin D1-dependent but ligand-independent transactivation by ER. These studies suggest that SRC coactivators may form complexes with cell cycle regulatory proteins to precisely control gene expression at different stages of the cell cycle.

The ability of cyclin D1 to enhance ER transactivation is dependent on a C-terminal region containing a LLXXXL motif, which resembles a motif in the C-terminal AF-2 helix of ER. Disruption of the LLXXXL motif of cyclin D1 impairs cyclin D1-dependent transcriptional activation of ER, although the mutant protein still binds to the unliganded ER. Interestingly, cyclin D1-dependent transactivation does not seem to rely on the AF-2 function of ER, and the SRC-1 dominant negative mutant containing only the C-terminal LXXLL motif markedly repressed the cyclin D1-induced activation of the ER AF-2 mutant. These data suggest a functional interaction between cyclin D1 and SRC-1. As expected, wild-type but not the LLXXXL mutant of cyclin D1 interacts with SRC-1 and AIB1 in a coimmunoprecipitation assay and such interactions appear to be direct. Interestingly, the LXXLL motifs of SRC-1, which mediate the interaction between SRC-1 and nuclear receptors, also interact with cyclin D1. Motif iii of SRC-1 appears to interact with cyclin D1 preferentially (Zwijsen *et al.*, 1998), in contrast to the prefer-

FIG. 14. SRC coactivators are histone acetyltransferases. (A) Mapping of the HAT domain of SRC-1. The position of domains for the bHLH, PAS, serine/threonine (S/T)-rich, glutamine(Q)/rich and dominant-negative (DN) regions are as indicated. White and black bars denote regions of SRC-1 without and with HAT activity, respectively, as determined by the filter-binding HAT assay of GST-SRC-1 fusion proteins. The indicated portions of SRC-1 were expressed as GST fusion proteins in *Escherichia coli* (383–568, 383–841, 782–1139, 1107–1441), yeast (1–399, 1216–1441), or insect cells (383–841) and subsequently purified using glutathione-Sepharose beads. The GST control protein was expressed in *E. coli*. About 2 pmol of GST control or indicated GST-SRC-1 fusion proteins was tested for the ability to acetylate free histones in a filter-binding assay using [³H]acetyl-CoA. (B) SRC-1 preferentially acetylates amino-terminal peptide tails of histones H3 and H4. Acetylation of histone N-terminal peptides by GST-SRC (1107–1441) was assessed by measuring ³H-acetate incorporation using the filter binding assay. For each peptide substrate and H1/H5-stripped chicken mononucleosomes, incubations with 2 pmol GST (white bars) or GST-SRC (1107–1441) (black bars) were done in parallel. Sites where N-acetyllysine was incorporated during peptide synthesis in order to mimic sites that are acetylated *in vivo* are indicated by (Ac). All peptides were MAP reagents, except diacetyl(9/14)-H3 peptide, which was synthesized with a C-terminal cysteine. [Adapted by permission from Figs. 2 and 3 of Spencer, T. E., *et al.* (1997). Steroid receptor coactivator-1 is a histone acetyltransferase. *Nature* 389, 194–198.]

ence of motif ii for interaction with ER (Heery *et al.*, 1997). Therefore, various LXXLL motifs of SRC-1 may display specificity for protein-protein interaction with nuclear receptors and cyclin D1, permitting simultaneous interaction of SRC-1 with both cyclin D1 and ER. This observation may explain, at least partly, the multiplicity of the LXXLL motifs in SRC coactivators. The ability of cyclin D1 to bind and activate ER and to interact with SRC-1 suggests an adaptor function for cyclin D1 between unliganded ER and SRC-1 in the absence of ligand. The formation of such a ternary complex also occurs on DNA-bound ER, suggesting that cyclin D1 can promote ligand-independent transactivation of ER by recruiting SRC coactivators to target genes. Physiologically, cyclin D1 mutant that fails to interact with SRC-1 appears to inhibit ER transactivation in breast cancer cells, suggesting an involvement of both cyclin D1 and SRC coactivators on ER-mediated transactivation in breast cancer cells.

The preceding observations suggest that cyclin D1 may provide a single site for interaction with LXXLL motif iii of the SRC coactivator in the absence of ligand. Upon ligand binding of ER, a second binding site is formed on ER for interaction with the LXXLL motif ii of the SRC coactivator. This model may partly explain the synergistic action of estradiol and cyclin D1 on ER activation. However, because unliganded ER also forms complexes with heat shock proteins and liganded ER binds to DNA as homodimer, the precise mechanism of synergism between cyclin D1 and SRC coactivators in ER activation remains unclear. Nevertheless, because cyclin D1 and AIB1 are overexpressed frequently in breast cancer cells, the synergistic action of cyclin D1 and SRC coactivator may have significant roles in ER-dependent cell growth and proliferation of breast cancer cells.

VI. SRC FUNCTION AND SPECIFICITY

The existence of three related SRC coactivators with similar property in transcriptional activation and steroid/nuclear receptor interaction suggests a redundant mechanism for coactivator function. In fact, all three SRC coactivators interact and activate multiple steroid/nuclear receptors. However, several studies have also provided evidence that each SRC coactivator may exhibit a specific mode of function to precisely control transcriptional activation mediated by steroid/nuclear receptors and other classes of transcription factors. This section discusses the possible function and specificity of members of the SRC coactivator family.

A. EXPRESSION PATTERNS

To understand the physiologic function of SRC coactivators, the expression patterns of each SRC coactivator have been analyzed and compared. The SRC-1 message was detected ubiquitously in many tissues (Li and Chen, 1997; Misiti *et al.*, 1998; Zhu *et al.*, 1996; Yao *et al.*, 1996). The expression of SRC-1 is relatively high in skeletal muscle, heart, brain, and pancreas, and low in lung, liver, and kidney (Fig. 15). In a Northern blot assay, two SRC-1 messages of distinct sizes were detected, with the longer form (8 kb) more abundant than the shorter form (7 kb). The identity of these two forms is currently unclear, but they likely represent the SRC-1a and SRC-1e isoforms, respectively. In contrast to the expression of SRC-1, expression of RAC3 (SRC-3) is highly restricted (Fig. 15). The relative abundance of TIF2 message in human tissues is similar to that of RAC3 (Li and Chen, 1997). Both TIF2 and RAC3 are highly expressed in placenta, uterus, mammary gland, pituitary, testis, heart, skeletal muscle, and pancreas, but at lower levels in brain, lung, liver, kidney, and bone marrow (Li and Chen, 1997; Chen *et al.*, 1997; Takeshita *et al.*, 1997; Suen *et al.*, 1998). Interestingly, mouse SRC-2 (GRIP1) and SRC-3 (p/CIP) were detected ubiquitously in many murine tissues, including lung, brain, heart, liver, and testis

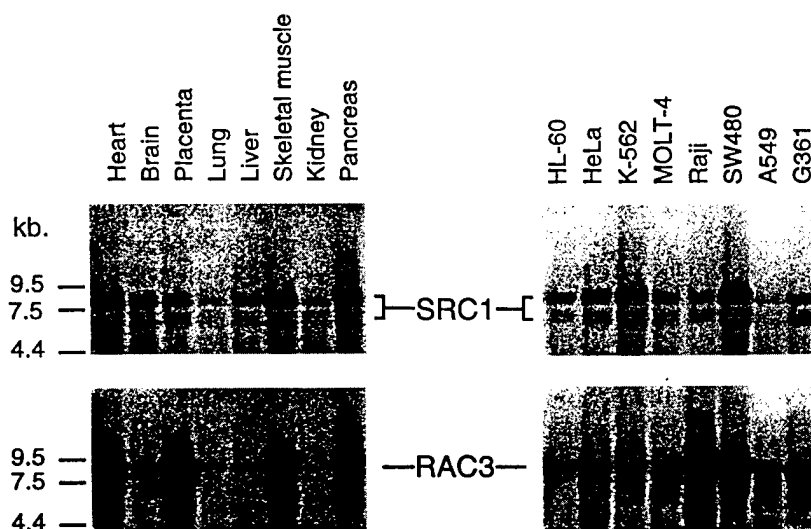


FIG. 15. Expression patterns of SRC-3 coactivators. Human multiple tissue (left) and cancer cell (right) Northern blots (Clontech Inc.) were sequentially hybridized with a ³²P-labeled RAC3 and SRC-1 probes.

(Torchia *et al.*, 1997). Therefore, the expression patterns of the SRC coactivators may differ among different species. For instance, TIF2 message is low in human kidney, liver, and lung, but the corresponding mouse tissues express high levels of TIF2. Similarly, SRC-3 appears to be ubiquitously expressed in mouse tissues (Torchia *et al.*, 1997), while expression of SRC-3 is highly restricted in human tissues (Li and Chen *et al.*, 1997; Chen, 1997). Consistent with the species-specific distribution, *Xenopus* SRC-3 is highly expressed in adult liver (Kim *et al.*, 1998), where SRC-3 is virtually undetectable in human. These results suggest that the expression of SRC coactivators may differ in different species, reflecting a potential functional difference for specific SRC coactivators in different species.

The expression levels of each SRC coactivator also vary significantly in different cancer cell types. SRC-3 is highly expressed in Burkitt's lymphoma Raji cells, and moderately expressed in epithelioid carcinoma HeLa cells, chronic myelogenous leukemia K-562 cells, colorectal adenocarcinoma SW480 cells, and the melanoma G361 cells (Chen *et al.*, 1997; Li and Chen, 1997; Misiti *et al.*, 1998). The cell-type expression pattern of SRC-2 (TIF2) is similar to that of SRC-3 with the highest expression in the Raji cells. In contrast, SRC-1 is expressed at high levels in K-562 and SW480 cells, with low levels in HL60, HeLa, MOLT-4, Raji, A549, and G361 cells. In addition, SRC-1 message was also detected in many other cell types, including GH3, AtT20, Rat1, NIH3T3, 293, COS7, CHO-K1, and CV-1, with relatively higher levels in the pituitary GH3 cells (Misiti *et al.*, 1998). Both SRC-1a and SRC-1e were also detected in many cell lines analyzed by Rnase protection assay (Kalkhoven *et al.*, 1998). These studies indicate that SRC coactivators are widely expressed in different cell types, suggesting a wide spreading function for SRC coactivators. The differential expression of SRC coactivators suggests that each member of the SRC family might serve as a primary coactivator for a subset of receptors in a given tissue or cell type. Currently, which coactivator is involved in a particular hormonal signaling pathway remains to be determined.

It is likely that the expression level of both the receptors and coactivators and possibly their interactions with other transcriptional regulators will play an important role to control a precise level of gene expression in response to specific hormones. Frequently, the expression of receptor gene is autoregulated by the hormone that binds to and activates the receptor. For instance, expression of RAR β is upregulated by RAR ligand RA (de-Thé *et al.*, 1989). Interestingly, the expression of the coactivator RAC3 appears to be upregulated by RA as well (Li and Chen, 1997). Similarly, T3 treatment also produces an increase in SRC-

1 mRNA level in GH3 cells, as well as in the pituitary gland of adult rats (Misiti *et al.*, 1998). Therefore, it is possible that autoregulation of expression of SRC coactivators may add another level of complexity for cells to control gene expression induced by hormones (Fig. 16).

B. COACTIVATOR FUNCTION

Many studies have established the function of SRC coactivators for enhancing ligand-dependent transcriptional activity of steroid/nuclear receptors (Jeyakumar *et al.*, 1997; Henttu *et al.*, 1997; Zhu *et al.*, 1996; McInerney *et al.*, 1996; Smith *et al.*, 1996). Transient transfection has been widely utilized to show the coactivation function of SRC coactivators. For instance, transfection of SRC-1 enhances progesterone-stimulated transactivation by PR, while transfection of SRC-1 has little effect on RU-486 antagonist bound PR (Oñate *et al.*, 1995). It has been shown that overexpression of SRC-1 results in enhancement of ER, GR, TR, PPAR, and RXR transcriptional activities, but has no effect on E2F- or forskolin-stimulated transcription (Oñate *et al.*, 1995; DiRenzo *et al.*, 1997; Zhu *et al.*, 1996). Overexpression of SRC-1 can also reverse the inhibitory effect of E2 on R5020-stimulated transcription, and the C-terminal receptor-interacting domain alone inhibits hormone-stimulated PR and TR transactivation (Oñate *et al.*, 1995). Similarly, SRC-2 (GRIP1/TIF2) and SRC-3 (RAC3/p/CIP/ACTR/AIB1/TRAM-1) also ex-

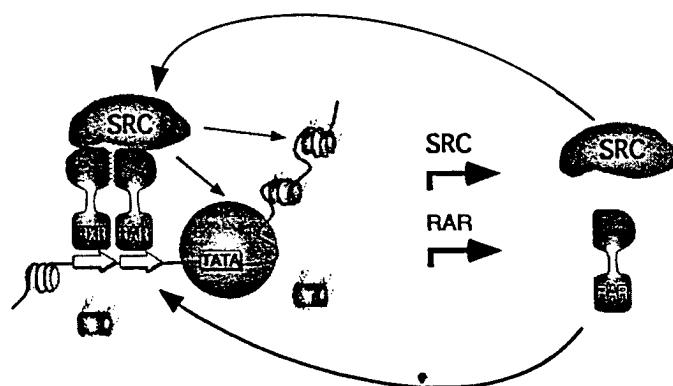


FIG. 16. Model of coactivator autoregulation. After ligand binding, the RXR-RAR heterodimer recruits a coactivator complex that contains members of the SRC family proteins, CBP/p300 and P/CAF. Because both RAR and RAC3 transcripts are elevated by RA treatment, the increased concentration of the two proteins should further amplify the transcriptional responses, leading to a high level of gene induction.

hibit similar coactivation function. Although the relative level of enhancement depends on experimental conditions, these studies suggest that SRC coactivators are limiting cofactors shared by members of the steroid/nuclear receptor family.

The function of SRC coactivators in transcriptional activation by steroid/nuclear receptors and other classes of transcription factors has also been extensively analyzed by microinjection assay. Injection of anti-SRC-1 (NCoA-1) IgG completely inhibits RA stimulated transcription (Fig. 6) (Korzus *et al.*, 1998). Interestingly, such IgG-dependent inhibition could be rescued by coinjection of a NCoA-1 expression vector. Similarly, anti-SRC-1 IgG also inhibits transactivation by troglitazone-stimulated PPAR γ (Westin *et al.*, 1998), estradiol-stimulated ER, triac-stimulated TR, or progesterone-stimulated PR (Torchia *et al.*, 1997). In contrast, injection of anti-SRC-1 IgG had no effect on transactivation from Sp1-dependent or CMV promoters (Torchia *et al.*, 1997), or from cAMP-stimulated CREB or interferon γ -stimulated STAT-1-dependent promoters (Korzus *et al.*, 1998), suggesting the specificity of this assay. Paradoxically, injection of anti-NCoA-2 (SRC-2) IgG had no effect on RA-dependent transcription (Torchia *et al.*, 1997), contradicting transient transfection data (Hong *et al.*, 1997; Voegel *et al.*, 1998). Injection of anti-p/CIP (SRC-3) IgG appears to have a profound inhibitory effect on not only all steroid/nuclear receptors tested (RAR, ER, TR, PR), but also on interferon γ and cAMP-dependent transcriptional activation (Torchia *et al.*, 1997; Korzus *et al.*, 1998). These studies suggest a broader role for p/CIP (SRC-3) in different signaling pathways than SRC-1 and SRC-2, consistent with the hypothesis that p/CIP is a component of the CBP/p300 cointegrator complex (Torchia *et al.*, 1997).

By performing IgG microinjection together with a rescuing expression vector for either wild-type or mutant coactivator, the domain requirement and functional redundancy of the three SRC coactivators have been revealed. First, coinjection of wild-type SRC-1 expression vector efficiently restores RA-dependent transcription abrogated by anti-SRC-1 IgG (Fig. 6). It appears that, in addition to SRC-1, SRC-2 (NCoA-2) but not SRC-3 (p/CIP) also restores the anti-SRC-1 IgG-inhibited transcription from a RA-dependent promoter (Torchia *et al.*, 1997), suggesting a functional redundancy between SRC-1 and SRC-2, but not with SRC-3. Interestingly, the inhibition of RA-dependent transcription by p/CIP IgG could only be rescued by coinjection of both p/CIP and CBP expression vectors. Coinjection of individual expression vector for NCoA-1, NCoA-2, or even p/CIP or CBP could not restore RA-dependent transcription abrogated by anti-p/CIP IgG (Torchia *et al.*, 1997). These studies are consistent with the idea that both NCoA-1 and

the CBP/p300/p/CIP complex are independently required for gene activation by steroid/nuclear receptors (Fig. 17). In addition, both LXXLL motif ii and motif iii mutants of SRC-1 were unable to restore IgG-inhibited transactivation by RAR, while only motif ii but not motif iii mutation failed to restore ER transactivation. These data indicate that both motif ii and iii are essential for transactivation by RAR, while motif iii is not required for transactivation by ER but motif ii is essential (Torchia *et al.*, 1997). These results also indicate a differential requirement for each LXXLL motif in transcriptional activation by specific steroid/nuclear receptors. Furthermore, the dominant negative effect of either receptor-interacting or transcriptional activation domain of p/CIP alone has also been demonstrated by the microinjection assay. Coinjection of expression vector for p/CIP fragment between amino acids 547 and 1084 inhibited RA-dependent transcription. In contrast, injection of expression vector for p/CIP fragment 947 to 1084 inhibits interferon g-stimulated transcription, which could not be restored by coinjection of CBP (Torchia *et al.*, 1997).

The presence of multiple HAT components of the SRC-CBP/p300-P/CAF coactivator complex raises a question about the requirements for specific HAT activities in transcriptional activation by steroid/nuclear

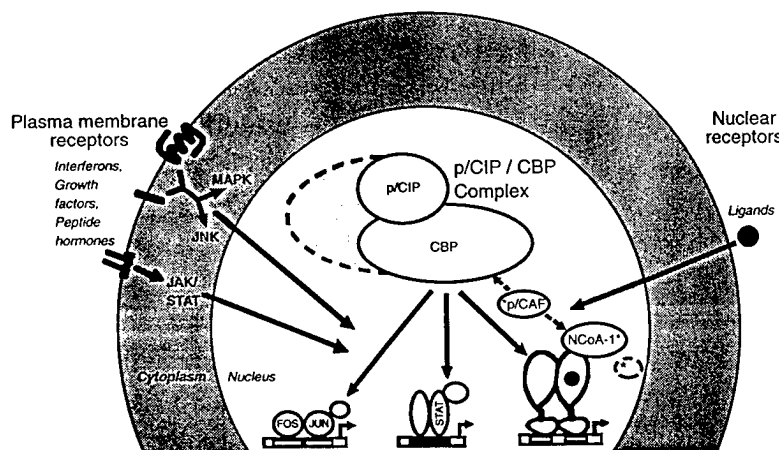


FIG. 17. Model of SRC coactivator function in different signaling pathways. Several signal-transduction pathways that are mediated by specific transcription factors require a functional SRC/CBP/p300 coactivator complex, and potentially p/CAF, with each partner being required, but not sufficient, to mediate transcriptional effects. [Adapted by permission from Fig. 6 of Torchia, J., *et al.* (1997). The transcriptional co-activator p/CIP binds CBP and mediates nuclear receptor function. *Nature* 387, 677–684.]

receptors. This question was also addressed by the microinjection assay. First, P/CAF was shown to be essential for RAR, TR, and ER-dependent transcriptional activation as microinjection of anti-P/CAF IgG abrogated all transactivation events (Korzus *et al.*, 1998). Similarly, microinjection of anti-CBP IgG also inhibits transcription activities of steroid/nuclear receptors, suggesting that all three classes of coactivators are required for steroid/nuclear receptor function. The requirements for specific acetyltransferase activities of these coactivators were then analyzed by coinjecting rescuing vector for either wild-type or acetylation defective mutants. The results show that only the HAT activity of P/CAF, but not CBP or SRC-1, is required for RAR-mediated transcriptional activation (Korzus *et al.*, 1998). Consistently, the HAT domain of the SRC coactivators is not essential for transcriptional activation by the coactivators (Chen *et al.*, 1997; Voegel *et al.*, 1998). Therefore, although the SRC coactivators are necessary for optimal transcriptional activation by steroid/nuclear receptors, the role of their HAT activity in transcriptional activation remains unclear.

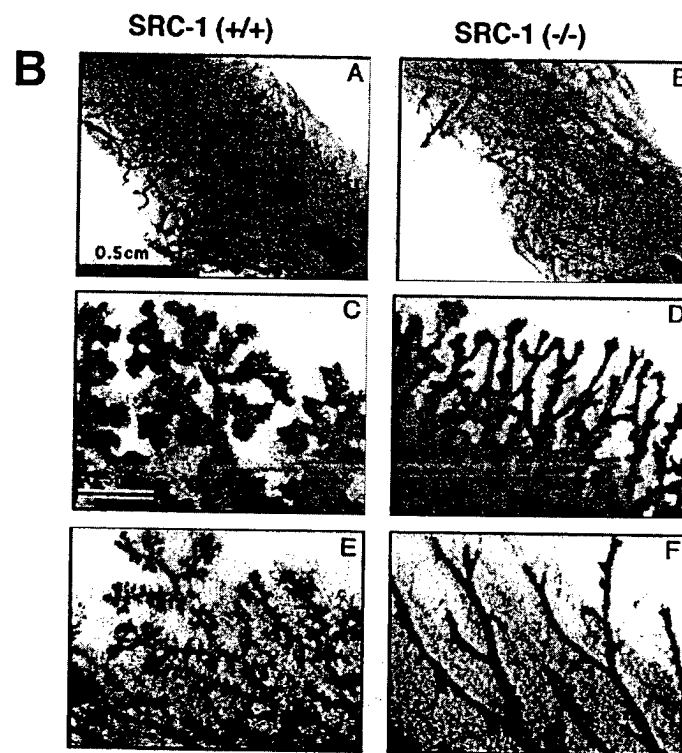
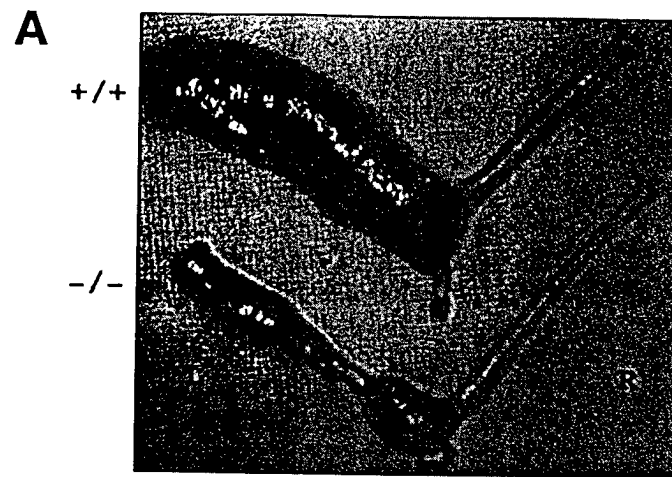
Similar to steroid/nuclear hormones, transforming growth factor β (TGF- β) also regulates cell proliferation and differentiation. Binding of TGF- β to cell surface receptor induces phosphorylation of SMAD2 and SMAD3, which are members of the SMAD family of transcription factors. The phosphorylated form of SMADs forms stable complexes with SMAD4 and these complexes translocate into nucleus where they activate transcription. Recently, TGF- β has been shown to act cooperatively with vitamin D₃, indicating a cross-talk between these two signaling pathways (Yoshizawa *et al.*, 1997; Takeshita *et al.*, 1998). The mechanism of synergism between TGF- β and vitamin D₃ appears to be mediated by SMAD3, but not SMAD2 (Yanagisawa *et al.*, 1999). SMAD3 interacts directly with VDR in a ligand-dependent manner *in vivo*, and this interaction is mediated through the NH₂-terminal Mad homology 1 (MH1) region of SMAD3 and a middle region of the ligand-binding domain of VDR. Interestingly, SMAD3 acts synergistically with SRC-1 to enhance transactivation of VDR (Yanagisawa *et al.*, 1999). It appears that interaction of VDR with SRC-1 is required for the ligand-dependent interaction with SMAD3, since an SRC-1 mutant lacking nuclear receptor interacting motifs inhibits the interaction of VDR with SMAD3. Although, SMAD3 does not appear to interact directly with SRC-1 or TIF2, certain SRC-1-stabilized ligand-dependent conformational changes in VDR may be required for SMAD3 interaction. Alternatively, activation of SMAD3 by TGF- β receptor-mediated phosphorylation may be required for interaction with SRC coactivators. It remains to be established whether the functional interaction be-

tween SMAD3 and SRCs plays a role in other TGF- β -mediated signaling pathways.

C. SRC-1 FUNCTION IN MICE

The *in vivo* biological function of the coactivator SRC-1 has been assessed in mice by gene targeting (J. Xu *et al.*, 1998; Qi *et al.*, 1999). In one study (J. Xu *et al.*, 1998), the endogenous SRC-1 gene was targeted by a vector that deletes all known SRC-1 functional domains except the N-terminal bHLH-PAS region. Although the bHLH-PAS domain is highly conserved among SRC family proteins, it is not essential for transcriptional coactivation by SRC-1. In the knockout mice, both the heterozygous and homozygous mice appear normal and indistinguishable from wild-type mice. Both male and female homozygotes are fertile and develop at a similar rate as the wild-type mice. However, detailed analysis of steroid action in target organs including uterus, prostate, and mammary gland revealed that SRC-1 function is in fact required for maximal response of these organs to steroids *in vivo* (Fig. 18).

First, the uterine response to progesterone appears significantly impaired in SRC-1 null mice. This was measured in ovariectomized mice treated with a high dose of progesterone and a low dose of estrogen, followed by mechanical traumatization (decidual stimulation) of one uterine horn. In wild-type mice, the uterine horn increases in size in response to decidual stimulation. In contrast, the uterine response in SRC-1 null mutant is significantly reduced (2.5-fold) (Fig. 18). Similarly, estrogen-induced uterine growth in SRC-1 null mutants is also significantly reduced. These data suggest that SRC-1 is required for maximal uterine response to steroid hormones *in vivo*. Uterine response to mechanical traumatization is a progesterone receptor (PR)-dependent process; therefore, SRC-1 may be required for efficient transcriptional activation by PR, consistent with its coactivation function in tissue culture cells (Oñate *et al.*, 1995). In addition to progesterone-dependent uterine response, androgen-dependent growth of prostate and testes, and estrogen and progesterone-dependent growth of mammary gland are also significantly inhibited in SRC-1 mutant mice compared to wild-type mice. In castrated male mice with regressed prostates, testosterone-stimulated prostate growth is significantly reduced in SRC-1 mutant mice. Consistently, the size of testes is also smaller in SRC-1 null mutants. Furthermore, the development of mammary ducts and alveoli in virginal and pregnant mice, respectively, are both retarded in SRC-1 null mutants (Fig. 18). Mammary gland development in ovariec-



tomized mice in response to estrogen and progesterone treatments is also significantly affected. Furthermore, estradiol, progesterone, and testosterone concentrations in female null mutants are 1.2 and 1.5 times those in wild-type animals, respectively, consistent with the phenomenon of endocrine feedback regulation. Analysis of the expression of other SRC coactivators in the SRC-1 null mutant mice revealed an elevated level of TIF2, suggesting that other SRC coactivators may redundantly or partially replace the lost function of SRC-1. The partial hormonal response due to impairment of coactivator function might explain certain partial-hormone-resistance syndromes.

The mouse SRC-1 gene was also targeted to replace its central nuclear receptor interacting domain with phosphoglycerate kinase-neomycin gene in another study (Qi *et al.*, 1999). In this case, a correct gene-targeting event would result in a protein that lacks the three critical LXXLL motifs required for interaction with liganded receptors. Similar to the other study (J. Xu *et al.*, 1998), the homozygous SRC-1^{-/-} mice were viable and exhibited no apparent morphologic abnormalities. Both male and female homozygous mice grew normally and were fertile. Extensive analysis of the PPAR α B ligand-mediated responses *in vivo* suggests that SRC-1 is not required for PPAR α -mediated transcriptional activation. For instance, the SRC-1 null mice response normally to peroxisome proliferators, such as ciprofibrate and Wy-14,643, which induce liver cell proliferation and hepatic peroxisome proliferation. There were also no effects on the expression of PPAR α -regulated, fatty acid-metabolizing enzymes in the liver. Because this targeting event may allow expression of a truncated SRC-1 mutant, a

FIG. 18. Uterine and mammary gland development in SRC-1^{-/-} mutant mice. (A) Uterine responses to a decidual stimulus were measured in wild-type (+/+) or SRC-1 null mutant (-/-) females. Eight-week-old females were ovariectomized on day 0, treated with estradiol (0.1 μ g per mouse per day) from day 10 to day 12, and treated with progesterone (1 mg per mouse per day) and estradiol (6.7 ng per mouse per day) from day 16 to day 23. Mechanical decidualization in the left uterine horn was done 6 h after hormone injection on day 18. The whole uterus was dissected 6 h after hormone injection on day 23. (B) The fourth pair of mammary glands from 8-week-old virgins with the indicated SRC-1 genotypes (A and B). The ducts and alveolar structures of the fourth pair of mammary glands from mice pregnant for the first time with the indicated genotypes (C and D). The mammary ducts and alveolar structures of the fourth pair of mammary glands from 13-week-old females treated with progesterone and estradiol. Eight-week-old females were ovariectomized one day 0 and then treated with progesterone (1 μ g per mouse per day) and estradiol (50 mg per mouse per day) from day 14 to day 34 (E and F). [Reprinted with permission from Xu, J., *et al.* (1998). Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene. *Science* 279, 1922-1924. Copyright © 1998 American Association for the Advancement of Science.]

functional SRC-1 mutant might still exist in the homozygous mice. Nonetheless, there is no evidence for such a truncated protein and SRC-1 may indeed be nonessential for PPAR α -mediated transcriptional response *in vivo*. Alternatively, loss of SRC-1 function might be fully compensated for by other nuclear receptor coactivators. Reciprocal examination of the steroid hormone responses and PPAR α function in different SRC-1 null mutant strains might help to clarify the requirement of SRC-1 in steroid/nuclear receptor function in mice. Apparently, additional studies are necessary to fully understand the role of various coactivators by generating mutant mice with defects in one or more coactivator functions.

VII. SRC COACTIVATORS AND HUMAN DISEASES

A. MOZ-TIF2 FUSION IN ACUTE MYELOID LEUKEMIA

Recently TIF2 was found in a search for genes involved in inv(8)(p11q13)-associated acute myeloid leukemia (AML) (Carapeti *et al.*, 1998). This subtype of AML contains blast cells of a monocytoid phenotype that have pronounced erythrophagocytic activity. This AML subtype is typically associated with the t(8;16)(p11;p13) translocation, and occasionally with the t(8;22)(p11;q13), t(8;19)(p11;q13), and inv(8)(p11;q13) translocations (Mitelman *et al.*, 1997). The genes involved in the t(8;16) have been identified as the MOZ gene at 8p11 fused to the coactivator CBP gene at 16p13 (Borrow *et al.*, 1996). Although the precise function of the MOZ gene is unknown, it contains a PHD/LAP domain involved in protein-protein interaction, and a histone acetyltransferase homologous domain. Because CBP is also a histone acetyltransferase, the mechanism of leukemogenesis in patients with the t(8;16) may involve aberrant chromatin remodeling due to abnormal histone acetylation. Although TIF2 itself has not been shown to have histone acetylation activity, other TIF2-related SRC coactivators possess histone acetylation function, reinforcing the supposition that abnormal chromatin acetylation may cause leukemia. In the inv(8)(p11q13) translocation, the MOZ-TIF2 fusion retains the N-terminal PHD finger and HAT domains of MOZ, along with the C-terminal CBP-interacting domain and the putative HAT domain of TIF2 (Fig. 19). The fusion does not contain the bHLH-PAS or the steroid/nuclear receptor interacting domains of TIF2. Therefore, the HAT activity of TIF2 or its associated protein CBP might overstimulate expression of genes normally regulated by MOZ (Fig. 20). In addition to CBP and TIF2, other coactivators are also found associated with leukemogenesis or other

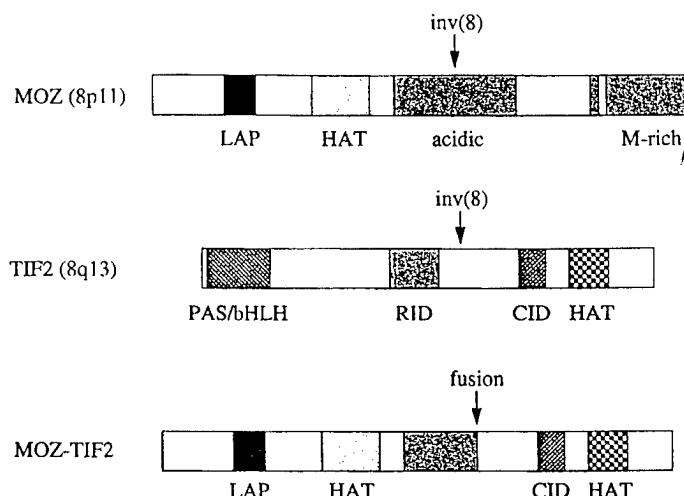
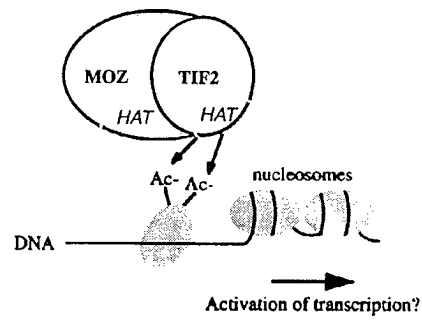
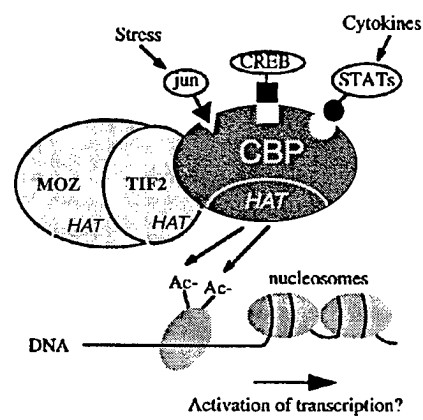
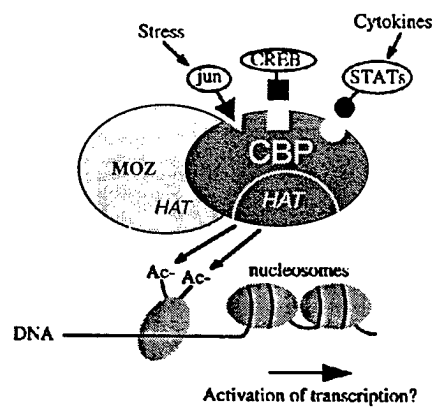


FIG. 19. Schematic representation of MOZ, TIF2, and MOZ-TIF2 fusion proteins. Domains are indicated as follows: LAP, leukemia-associated protein; HAT, histone acetyltransferase; M-rich, methionine-rich; CID and RID, putative CBP and nuclear receptor interacting domains based on homology with SRC-1. The MOZ-TIF2 fusion retains the LAP finger and HAT homology domains of MOZ, along with the CID and HAT domains of TIF2. [Reproduced by permission from Fig. 4 of Carapeti, M., *et al.* (1998). A novel fusion between MOZ and the nuclear receptor coactivator TIF2 in acute myeloid leukemia. *Blood* 91, 3127-3133.]

cancer types. For instance, p300 is fused to the MLL gene in AML with the t(11;22)(q23;q13), ARA70 is fused to RET in human thyroid papillary carcinoma, and TIF1 fused to B-RAF in the mouse hepatoma-derived oncogene T18. These observations suggest that transcriptional coactivators such as those for nuclear receptors may be widely involved in malignancy.

B. AIB1 GENE AMPLIFICATION IN CANCERS

Gene amplification is frequently associated with human cancers for selective overexpression of a subset of genes essential for supporting tumor growth. In breast cancer, several chromosomal regions are commonly amplified, including regions in the long arm of chromosome 20. In a search of target genes amplified from chromosome 20q in breast cancer, AIB1 was cloned by chromosome microdissection and hybrid selection, and mapped to 20q12 (Anzick *et al.*, 1997). Accordingly, AIB1 gene was found highly amplified (>20-fold) in three ER-positive breast carcinoma cell lines (BT-474, MCF-7, and ZR75-1) and in one ovarian

A**B****C**

carcinoma cell line (BG-1) (Fig. 21) (Anzick *et al.*, 1997). In contrast, both SRC-1 and SRC-2 are ubiquitously expressed at low levels in all breast cancer lines analyzed. AIB1 amplification also occurs in primary breast tumors (9.5%), although the amplification levels are not as high as the cell lines (Anzick *et al.*, 1997). As expected, AIB1 gene amplification causes overexpression of AIB1 mRNA and protein. Interestingly, 58% of the mammary tumors that show no AIB1 gene amplification exhibit overexpression of AIB1, as compared with normal mammary epithelium. This suggests that overexpression of AIB1 in breast cancer cells may occur through mechanisms other than gene amplification. In a recent screening of 1157 breast and 122 ovarian tumors by Southern blotting, AIB1 amplification was found in 4.8% of breast cancers and 7.4% of ovarian cancers (Bautista *et al.*, 1998). The degrees of amplification range from 2- to 8-fold in breast tumors and 2- to 10-fold in ovarian tumors. These results indicate that the frequency and level of AIB1 amplification appear higher in ovarian tumors than in breast tumors. In breast tumors, AIB1 amplification appears to correlate positively with either ER or PR expression. In addition, AIB1 amplification is more frequently observed in large tumors (>2 cm) and seems to correlate with MDM2 and FGFR1 amplifications. In contrast, no correlation was found with cyclin D1, Erb-B2, or Myc amplifications. MDM2 is the main repressor of the tumor suppressor p53, thus amplification of MDM2 may result in p53 inactivation. The FGFR1 is a class IV tyrosine kinase receptor that is preferentially activated by FGFs and amplified in 10–15% of breast tumors. The coamplification of AIB1 with MDM2 and FGFR1 suggests possible cooperative pathways of oncogenic activation in breast cancers. Interestingly, although cyclin D1 amplification in breast cancer is clearly associated with ER positivity, cyclin D1 is not coamplified with AIB1. This observation suggests that AIB1 and cyclin D1 amplifications correspond to a distinct subset of ER-positive breast tumors. Recently, cyclin D1 has been shown to interact

← FIG. 20. Hypothetical models of the mode of action of the MOZ-TIF2 fusion protein. (A) TIF2 may directly modulate the transcriptional activity of genes normally regulated by MOZ through the addition or removal of histone acetyl (Ac) groups by its HAT domain. (B) The TIF2 moiety may serve as a bridge between MOZ and CBP, and it is the HAT or other activities of CBP that leads to leukemogenic alterations in gene expression. Chromatin-associated CBP may be responsive to other cellular signals such as those mediated by Jun, CREB, or STAT proteins. (C) The MOZ-CBP fusion in the t(8;16),7, which is associated with a strikingly similar leukemia cell phenotype to that seen in cases with the inv(8). [Reproduced by permission from Fig. 5 of Carapeti, M., *et al.* (1998). A novel fusion between MOZ and the nuclear receptor coactivator TIF2 in acute myeloid leukemia. *Blood* 91, 3127–3133.]

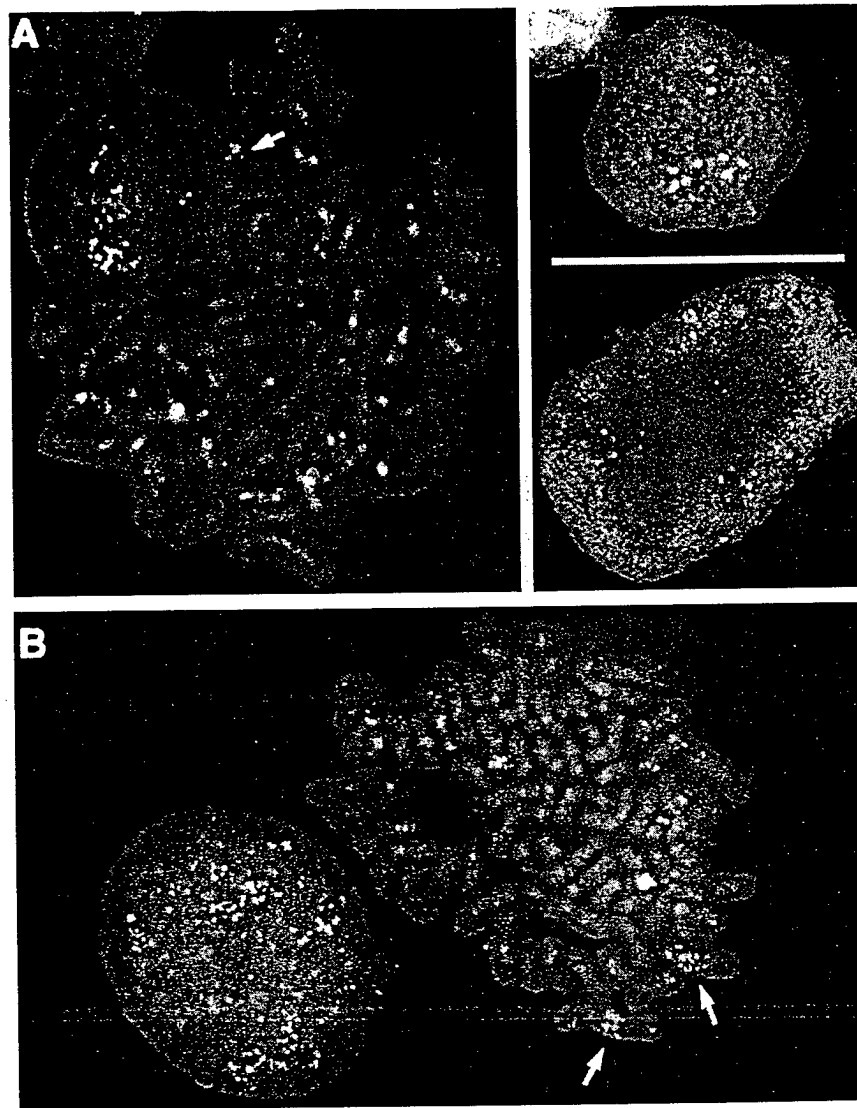


FIG. 21. Amplification of a SRC coactivator in breast cancers. Bicolor FISH analysis demonstrates AIB1 gene amplification in breast cancer cell line ZR75-1 (A), ovarian cancer cell line BG-1 (B), and two uncultured breast cancer samples (C). Intrachromosomal amplification of AIB1 (arrows) is apparent in metaphase chromosomes of ZR75-1 and BG-1, and numerous copies of AIB1 are resolved in the adjacent interphase nuclei. [Reprinted with permission from Anzick, S. L., *et al.* (1997). AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science* 277, 965-968. copyright © 1997 American Association for the Advancement of Science.]

with both ER and SRC coactivators (SRC-1 and AIB1) in a ligand-independent manner (Zwijnsen *et al.*, 1998). Because the frequency of cyclin D1 amplification correlates with the levels of ER expression, it is conceivable that synergistic action among ER, cyclin D1, and AIB1 may be an indicator of breast tumors.

In addition to breast and ovarian cancers, AIB1 gene amplification and overexpression have also been observed in pancreatic carcinoma (Ghadimi *et al.*, 1999). The incidence of pancreatic cancer is about 0.01 percentage in USA, which contributes to about 20 percentage of cancer deaths, due to poor prognosis of this disease. Cytogenetic studies revealed recurring chromosomal gains on several locations, including chromosome 20q where AIB1 gene is located. Fluorescence *in situ* hybridization (FISH) analysis found that the AIB1 gene is amplified in six out of nine pancreatic carcinoma cell lines, which partially correlate with overexpression of AIB1 mRNA (Ghadimi *et al.*, 1999). These results suggest that AIB1 gene amplification may occur frequently in human tumors and that steroid/nuclear receptors may regulate growth of cells that are not primarily controlled by endocrine stimuli. Alternatively, AIB1 may be involved in signaling pathways other than steroid/nuclear receptors, at least in part, due to its interaction with the general coactivator CBP/p300.

VIII. CONCLUSIONS

In conclusion, a novel family of transcriptional coactivators has been identified and shown to play a crucial role in transcriptional activation by steroid/nuclear hormone receptors and possibly other classes of transcriptional regulators. Detailed biochemical and structural analyses have revealed the molecular basis of protein-protein interaction between SRC coactivators and several liganded steroid/nuclear receptors. Additionally, transcription coactivation by SRCs has been linked to histone acetylation, partly by association with general transcriptional coactivators CBP/p300 and P/CAF. It is currently unknown whether these three SRC coactivators share redundant functions or form a protein complex to synergize transcriptional activation. It is important to note that at least two members of the SRC family are directly linked to human malignancies, consistent with a prevailing involvement of steroid/nuclear receptors in human cancers. Future studies are required for understanding the physiologic role of these coactivators in hormone action and the potential development of these genes as drug targets for treating human diseases.

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THE CONSTRUCT INDEPENDENCE OF PERCEIVED STRESS AND DEPRESSIVE SYMPTOMS

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Due to overlap in operational definitions, perceived stress measures are questioned as predictors of psychological outcomes, especially depressive symptoms. For example, the Perceived Stress Scale (PSS; Cohen et al., 1983) and the CES-D (Kohout et al., 1993) are highly correlated ($r's > .50$), as found in the present study ($n = 166$; $r = .63$; $p < .0001$). However, previous studies of the PSS have relied on principal component analyses with varimax rotation, (Cohen et al., 1983; Hewitt et al., 1992; Martin et al., 1995), analysis appropriate for data reduction but not for uncovering factor solutions, variances and covariances (correlations). We examined the factor solutions of the PSS and CES-D together, using exploratory factor analysis with oblique rotation to a partially specified target, computed by the program CEFA (Browne et al., 1998). Subjects were women (mean age 50) with breast cancer participating in a 5 year longitudinal study of cancer stress. Based on previous research, the CES-D has 4 factors (1-depressed affect; 2-positive affect; 3-somatic complaints, and 4-interpersonal problems) and the PSS has 2 factors (1-distress; 2-coping). We statistically combined the PSS and CES-D (6 factors) to simultaneously test the factor loadings for construct redundancy. We also conducted factor analyses for 4 and 5 (underfactored) and 7 factors (overfactored). Goodness of fit measures for the 4, 5, and 7 factor solutions were either unsatisfactory or demonstrated overfactoring. The 6 factor solution, however, demonstrated goodness of fit ($RMSEA = .058$) and factor loadings reflecting previous findings (4 factors for the CES-D and 2 for the PSS). Findings suggest that perceived stress and depressive symptom measures are not, necessarily, construct redundant and that perceived stress may be useful as a predictor of psychological outcomes.

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OBJECTIVE STRESSORS VS. SUBJECTIVE STRESS AND THEIR RELATIONSHIP TO DEPRESSIVE SYMPTOMS IN WOMEN WITH BREAST CANCER

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The relative importance of stress perceptions to psychological outcomes was explored. In particular, the relationship of objective stressors (life events) and subjective (perceived) stress to depressive symptoms was examined using a clinically relevant paradigm, stressed individuals vulnerable to the experience of depressive symptoms, namely women ($n = 166$; mean age 50) recently diagnosed and surgically treated (within the previous 3 months) for stage II or III breast cancer. Analyses controlled for alternative hypotheses including: sociodemographic (age, race, partner status, education, income), disease (stage, surgery type, time since surgery), and personality (neuroticism) factors. Using Hierarchical Multiple Regression, 51% of the variance in depressive symptoms was accounted for by the control variables (race, neuroticism), objective stressors (major financial difficulty or major conflict with children/grandchildren; Life Events Scale; Mathews et al., 1997), subjective cancer stress (Impact of Events Scale-IES; Horowitz et al., 1979), and subjective global stress (Perceived Stress Scale-PSS; Cohen et al., 1983). The squared semipartial correlations indicated that perceived stress (10%), cancer stress (8%), and race (1%) accounted for significant unique variance in the final model. While "stress" measures are correlated, these findings indicate that subjective stress measures are uniquely better predictors of depressive symptoms than objective measures. Further, a global perception of stress was a stronger predictor than perceived stress for a specific event. Although the difference between the contribution of the PSS and the IES was small (2%), this finding remains impressive considering the clinical importance of the event—recent cancer diagnosis and surgery. Clinical implications of these findings indicate that experiencing financial and/or family problems along with the already stressful experience of breast cancer may increase a woman's vulnerability to depressive symptoms.

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ANXIETY SENSITIVITY: ITS RELATIONSHIP TO MOOD, SOCIAL SUPPORT, AND SOMATIC SYMPTOMATOLOGY IN WOMEN POST-ADJUVANT TREATMENT FOR BREAST CANCER

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High anxiety sensitivity (AS), the fear of anxiety-related bodily sensations, is a risk factor in the development of anxiety and related disorders. AS, not previously examined in cancer survivors, may be associated with the prevalence and severity of long-term psychological and somatic outcomes (e.g., anxiety, memory/concentration difficulties, fatigue). We used the Anxiety Sensitivity Index (ASI), the Profile of Mood States (POMS), Perceived Support Scale for Family/Friends (PSS), and a medical assessment (SWOG) with 62 women (age $M = 51.52$ years; 90% Caucasian) assessed 18-months post-surgery and 9 months post-adjuvant treatment (chemotherapy and/or radiation) for stage II or III breast cancer. All participants were part of a 5-year longitudinal study. As expected, ASI scores were positively correlated with the Total Mood Disturbance and Tension, Depression, Anger, and Confusion subscales of the POMS ($p's < .05$). ASI scores were also positively associated with somatic symptomatology (SWOG; $r = .33$, $p < .01$), specifically neurologic/neurocentral complaints (e.g. disorientation, agitation, fatigue, depression/anxiety). Finally, ASI scores were negatively associated with PSS from friends ($r = -.36$, $p < .01$). ANOVA revealed those with highest ASI scores reported less social support from friends ($F(2,58) = 3.44$, $p < .05$) than those with the lowest ASI scores. These findings indicate that anxiety sensitivity may be used to identify those women at risk for experiencing long-term negative psychological, social, and somatic outcomes after active treatment for breast cancer. Furthermore, the identification and management of AS may be a useful and appropriate target of psychosocial interventions (e.g., relaxation) designed for breast cancer patients.

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ABSTRACT

Conducting Clinical Research with Breast Cancer Patients: Issues of Recruitment and Retention

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We are testing a biobehavioral model of cancer stress and disease course (Andersen, Kiecolt-Glaser, & Glaser, 1994) which includes psychological (stress and quality of life), behavioral (health behaviors and compliance), and biological (immune) data with a 5 year randomized clinical trial. Women with stage II or III breast cancer are randomized between psychological/behavioral intervention (lasting 1 year) and assessment only arms. Issues of recruitment and retention are vital if a trial such as this is to successfully answer empirical questions (e.g., are intervention groups associated with longer survival?). We currently have a refusal rate of 31.8% and a drop out rate of 6.6% at a mean participation of 16 months (range = 1-30; initial data with $n = 137$). We have identified reasons for refusal and termination of participation (e.g., stress, distance, etc.) and discuss various strategies (e.g., addressing obstacles to participation) that researchers may use in recruiting and retaining cancer patients in intensive randomized longitudinal clinical trials.

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Golden-Kreutz, D., Thornton, L., Wells, S., Frierson, G., Lawrence, H., & Andersen, B.L. Negative changes in quality of life with the stress of cancer diagnosis and treatment. (In prep., abstract). The biobehavioral model predicts that the stress surrounding the diagnosis and treatment of cancer will be related to a subsequent adverse impact on emotional quality of life (qol). Using a sample of 215 women recently diagnosed and surgically treated for regional (stage II or III) breast cancer, this relationship was examined at two time points, an initial assessment conducted post-surgery and the first follow-up assessment 4 months later. Two components of quality of life, emotional and physical, were assessed using the SF-36. Preliminary correlational analyses were conducted to test for non-stress correlates of quality of life, including sociodemographic and disease factors. These preliminary analyses indicated that significant correlates of lower emotional qol were younger age, minority racial status, and increased impact of disease on daily functioning as measured by the Karnofsky Performance Scale (KPS). Correlates of lower physical qol were increased age, minority racial status, lower annual family income, absence of significant other, lower years of education, employment, having a mastectomy, and a lower KPS score. Therefore, these variables were used as control variables for subsequent analyses. The construct of stress was broadly assessed including stressful life events in the year preceding diagnosis (i.e., major financial difficulty), intrusive thoughts and avoidant behaviors surrounding diagnosis/treatment (Impact of Events Scale, IES), and global stress (Perceived Stress Scale, PSS). Using Hierarchical Multiple Regression, 60% of the variance in emotional qol post-surgery was accounted for by the control variable (race- having minority status) and perceptions of cancer stress (IES) and global stress (PSS). In examining the unique variance accounted for by the variables using squared semi-partial correlations (i.e., the amount of variance accounted for by a variable if it had been entered last in the regression equation), global stress accounted for 20% of the variance with cancer stress accounting for 4% and minority status 1%. Using the same stress regression model to predict emotional qol at the 4 month follow-up, 28% of the variance was accounted for. Again, perceived stress accounted for the most unique variance (7%) followed by cancer stress (2%) and minority status (2%). Next, using Hierarchical Multiple Regression, 31% of the variance in physical qol post-surgery was accounted for by the control variable KPS (6% unique variance), age (3%), employment (3%), and mastectomy (2%). At the 4 month follow-up, 31% of the variance in physical qol was again accounted for with significant unique variance accounted for by age (7%), KPS (3%), PSS (4%), and IES (2%). These data confirm the hypothesized relationship in the biobehavioral model regarding the adverse effects of stress on emotional and physical quality of life. Specifically, we found that while stress is related to both immediate and later emotional qol, it plays a larger role in later physical qol.

Stress and Immune Responses After Surgical Treatment for Regional Breast Cancer

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Background: Adults who undergo chronic stress, such as the diagnosis and surgical treatment of breast cancer, often experience adjustment difficulties and important biologic effects. This stress can affect the immune system, possibly reducing the ability of individuals with cancer to resist disease progression and metastatic spread. We examined whether stress influences cellular immune responses in patients following breast cancer diagnosis and surgery. **Methods:** We studied 116 patients recently treated surgically for invasive breast cancer. Before beginning their adjuvant therapy, all subjects completed a validated questionnaire assessing the stress of being cancer patients. A 60-mL blood sample taken from each patient was subjected to a panel of natural killer (NK) cell and T-lymphocyte assays. We then developed multiple regression models to test the contribution of psychologic stress in predicting immune function. All regression equations controlled for variables that might exert short- or long-term effects on these responses, and we also ruled out other potentially confounding variables. **Results:** We found, reproducibly between and within assays, the following: 1) Stress level significantly predicted lower NK cell lysis, 2) stress level significantly predicted diminished response of NK cells to recombinant interferon gamma, and 3) stress level significantly predicted de-

creased proliferative response of peripheral blood lymphocytes to plant lectins and to a monoclonal antibody directed against the T-cell receptor. **Conclusions:** The data show that the physiologic effects of stress inhibit cellular immune responses that are relevant to cancer prognosis, including NK cell toxicity and T-cell responses. Additional, longitudinal studies are needed to determine the duration of these effects, their health consequences, and their biologic and/or behavioral mechanisms. [J Natl Cancer Inst 1998; 90:30-6]

A diagnosis of cancer and cancer treatments are objective, negative events in an individual's life. Although negative events do not always produce stress and a lowered quality of life, data from many studies document severe, acute stress at cancer diagnosis (1) and during recovery (2). The negative psychologic responses of individuals with cancer to the diagnosis and treatment are important in their own right because these responses are targets for cancer control efforts (3,4). In addition, data suggest that stress responses are accompanied by nonrandom (i.e., correlated) negative changes in a broad range of immune responses. This study examines from a biobehavioral perspective whether stress influences cellular immunity in women with breast cancer after diagnosis of breast cancer and during the postsurgical period (5).

Meta-analyses (6,7) suggest that psychologic stress and the experience of life stressors are reliably associated with negative immune alterations in noncancer subjects; i.e., "higher" levels of stress (e.g., self-reports of stress or negative affects, such as sadness or clinical diagnoses of depression) are related quantitatively and functionally to "reduced" cellular immune responses, such as lowered natural killer (NK) cell lysis. This effect has been found regularly for individuals in the midst of chronic stressors, and some of the largest responses and

changes have been found for lengthy stressors and those that have interpersonal components.

Illustrative data come from Kiecolt-Glaser, Glaser, and colleagues (8-11), who have followed individuals during the long, stressful experience of giving care to a spouse diagnosed with Alzheimer's disease. Not surprisingly, caregivers report high levels of distress and negative affect as they cope with their relative's difficult behavior and mental deterioration (8). Moreover, these researchers have found, for example, that NK cells obtained from caregivers are less responsive to the cytokine recombinant interferon gamma (rIFN γ) and recombinant interleukin 2 (rIL-2) than are cells obtained from matched community control subjects (9). In addition, these highly stressed subjects have a poorer proliferative response to mitogens (8), exhibit substantial deficits in the antibody and virus-specific T-cell responses to an influenza virus vaccine (10), and demonstrate stress-related defects in wound repair (11).

There are fewer data on the relationship between stress and immunity among cancer patients. Levy et al. (12) reported on these relationships in 66 women with stage I or II breast cancer 3 months after treatment (lumpectomy or mastectomy with or without adjuvant therapy). In ad-

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dition to finding that estrogen receptor status predicted NK cell lysis, these researchers found that social support—a variable hypothesized to *reduce* stress—contributed significantly to a regression model predicting *higher* NK cell activity. These findings suggest that how a person responds to stress may also influence how stress, in turn, influences the immune response.

There is considerable evidence that patients with cancer express abnormal cellular immune responses; these abnormal responses have been found in patients with many different types of cancer (13–15), including breast cancer (16,17). Stressors are not generic, and they would not be expected to have identical physiologic outcomes. So too, the immune response involves a cascade of responses and events that can occur over time. For these reasons, we used a homogeneous breast cancer subject sample and timing of assessment to test the relationship between stress and several components of the cellular immune response, including NK cell and T-cell functions.

Women who had been diagnosed with breast cancer and who had undergone surgery for the breast cancer were studied before they began adjuvant therapy. Since we were interested in the contribution of stress in predicting an immune response above and beyond known correlates, we controlled for naturally occurring factors in our statistical analyses that affect the immune responses—specifically, age, disease stage (lymph node status), and recovery (days since surgery) (18). Because the immune system contains a considerable amount of redundancy, we focused on three components that would each provide important, but complementary, information.

First, we measured NK cell lysis. We chose to measure NK cell lysis because those cells are believed to act early in the immune response and they have been demonstrated to play an important role in immune surveillance against tumors and virally infected cells (19–21). Second, we measured the ability of the NK cells to respond to rIFN γ and rIL-2. It has been shown that lymphokine-activated killer (LAK) cells are highly cytotoxic against a wider variety of tumor cells than those lysed by resting NK cells (22), an effect also observed in patients with breast cancer (23). Finally, to obtain information on

the T-cell response, we measured the response of peripheral blood leukocytes (PBLs) to two mitogens—phytohemagglutinin (PHA) and concanavalin A (Con A)—and we induced proliferation by stimulating the T cells with a monoclonal antibody (MAb) to the T-cell receptor.

Subjects and Methods

Patient Eligibility and Data Collection

Participants were 116 women who had been diagnosed with invasive breast cancer and who were surgically treated within the last 4 months but who had not yet begun adjuvant treatment. Women were from 14 to 101 days (mean = 37 days; median = 33 days) after surgery for stage II (70%) or III (30%) invasive breast cancer. We used the American Joint Committee on Cancer and the International Union Against Cancer staging system. The women ranged in age from 31 to 84 years (mean = 52 years). Recruited consecutively from mid-1994 to early 1997, the majority (82%) were being treated at a National Cancer Institute-designated, university-affiliated Comprehensive Cancer Center, and the remainder (18%) were receiving treatment at local community hospitals. All women came to the General Clinical Research Center at the university where psychologic, behavioral, and medical data were collected and a 60-mL blood sample was taken from them. Assessments were conducted between 8:00 AM and 12:00 AM to reduce diurnal variability.

Stress Measure

The Impact of Event Scale (IES) (24) is a standardized self-report questionnaire used to examine intrusive thoughts ("I had dreams about being a cancer patient," "Other things kept making me think about cancer") and avoidant thoughts and actions ("I tried not to talk about it," "I was aware that I still had a lot of feelings about cancer, but I didn't deal with them") concerning cancer. Fifteen items are used, and women rate each event or feeling in terms of the frequency of occurrence (i.e., "not at all," "rarely," "sometimes," and "often") during the previous 7 days. Scores range from 0 to 75. For this sample, descriptive statistics were as follows: range, 0–65; mean = 26; median = 25; and standard deviation = 15.2. The scale has satisfactory reliability with internal consistency of .78–.82 and a 2-week test-retest reliability of .79–.89, respectively. The validity of the measure is suggested by data indicating that individuals who experience involuntary, distress-related thoughts following traumatic life events are also those who suffer the greatest negative effects psychologically [e.g., (21)].

Immune Assays

Blood cell separation. PBLs were isolated from 60 mL of venous blood by use of Ficoll gradients (Pharmacia Biotech, Inc., Piscataway, NJ). The isolated leukocytes were then washed in calcium- and magnesium-free phosphate-buffered saline and counted on a Coulter counter (Coulter Corp., Miami, FL). Aliquots of 8×10^6 isolated PBLs were suspended again in 0.8 mL of RPMI-1640 medium supplemented with 10% fetal bovine serum, 0.75%

sodium bicarbonate, 2 mM L-glutamine, and 10 μ g/mL of ciprofloxacin.

Quantification of total T lymphocytes, T-cell subsets, and NK cells. Isolated PBLs were absorbed with MABs conjugated to either fluorescein isothiocyanate or rhodamine according to the cell surface marker being studied: total T cells (CD3, fluorescein isothiocyanate), T4 subset (CD4, rhodamine), T8 subset (CD8, fluorescein isothiocyanate), and NK cells (CD56, rhodamine). All MABs were purchased from Coulter Corp. Briefly, 0.5×10^6 cells were incubated with the MAB for 15 minutes at room temperature. After the incubation, the cells were fixed, and the red blood cells were lysed with Opti-lyse C, a buffered solution containing 1.5% formaldehyde, according to the manufacturer's instructions (Coulter Corp.). Samples were analyzed with the use of a Coulter EPICS Profile II flow cytometer as described previously (8).

NK cell cytotoxicity. To determine NK cell activity, a microtiter ^{51}Cr -release cytotoxicity assay was used as described previously (9,25). The target cells used were K-562 cells, an NK cell-sensitive human myeloid cell line. Target cells, labeled overnight for 16 hours with ^{51}Cr , were placed in triplicate wells of 96-well V-bottom plates, and PBLs were added, resulting in effector-to-target (E:T) cell ratios of 100:1, 50:1, 25:1, 12.5:1, and 6.25:1.

NK cell response to cytokines. Procedures for treatment of PBLs with rIFN γ and rIL-2 involved preparing isolated PBLs at a concentration of 3×10^6 cells/mL in complete RPMI-1640 medium and then seeding the cells into three replicate tissue culture tubes (Falcon, Becton Dickinson and Co., Lincoln Park, NJ) at 6×10^6 cells per tube. Cells were incubated in complete RPMI-1640 medium alone or complete medium supplemented with 250 IU/mL rIFN γ or 60 IU/mL rIL-2 (Genzyme, Boston, MA). Cell suspensions were gently mixed and then incubated at 37°C in an atmosphere of 5% CO_2 for 65 hours. For the assay, triplicate aliquots of cell suspensions were placed in wells of V-bottom plates, with E:T cell ratios of 50:1, 25:1, 12.5:1, 6.25:1, or 3.13:1. In addition, six wells with target cells and medium only and target cells with detergent (5% sodium dodecyl sulfate in phosphate-buffered saline) were prepared to determine spontaneously released chromium and maximal lysis, respectively. The plates were centrifuged at 300g for 5 minutes at 20°C to bring the effector and target cells into close contact; they were then incubated at 37°C in an atmosphere of 5% CO_2 for 5 hours. After this incubation, the plates were centrifuged at 300g for 5 minutes at 20°C, 100 μ L of supernatant was collected from each well, and counts per minute were determined by use of a Beckman 9000 gamma counter (Beckman Instruments, Inc., Fullerton, CA) as described previously (9,26).

Blastogenic response to PHA, Con A, and MAB to the T3 receptor. The concentrations for PHA and Con A used were 2.5, 5.0, and 10.0 μ g/mL. To measure the blastogenic response to the MAB to the T-cell receptor, we used the following three dilutions of the purified MAB: 32:1, 64:1, and 128:1. For all three assays isolated, PBLs seeded in triplicate at 0.5×10^5 per well were incubated for 68 hours at 37°C in 96-well flat-bottomed plates and then labeled for 4 hours with MTS, i.e., 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt

(Promega Corp., Madison, WI) to measure proliferative response. Briefly, the MTS procedure is a nonradioactive calorimetric procedure that labels metabolically active cells via reduction of a colored substrate. The amount of proliferation was determined by optical density of the suspension in the well. Optical density determinations were performed by use of a Titertek Multiscan MCC microplate reader (Flow Laboratories, Inc., Finland) at a determination wavelength of 492 nm and a reference wavelength of 690 nm as has been noted (27,28).

Statistical Analyses

Preliminary analyses. Before conducting the principal analyses, we checked the data for the contribution of "nuisance" variables (covariates) that could potentially be related to psychologic stress, immune outcomes, or both [see (25) for a discussion]. The variables examined were measures of aspirin, alcohol, caffeine, and nicotine intake; amount of sleep; plasma albumin level (as an indicator of nutritional status); incidence of recent infectious illness; and the Karnofsky performance status rating. We examined the relationships between these variables and each of the three sets of outcome variables: NK cell lysis, ability of NK cells to respond to rIFN γ and rIL-2, and the blastogenic response of PBLs to Con A, PHA, and the T3 MAb. Analysis of variance was used for the categorical independent variables, and simple correlations were used for numerically scaled independent variables.

Screening of these potential covariates involved examination of the relationships between 11 covariates and 20 dependent variables, or a total of 220 bivariate associations. Of these 220 associations, 15 were found to be statistically significant at .05 significance level. This number of significant effects is only slightly more than would be expected by chance alone (i.e., $220 \times .05 = 11$). Inspection of the significant relationships showed that many of them were attributable to the influence of a few outliers in the data. To be conservative, all of the regression analyses described below were run twice, once including and once excluding those covariates that had significant bivariate associations with the relevant dependent variables. In no case were results of the regression analyses significantly altered by the inclusion of the covariates. Given this fact and the consistently weak relationships of the covariates to the dependent variables, we do not report further results involving the covariates.

Principal analyses. The principal analyses assess the relationship between the IES measure of psychologic stress and the following three sets of outcome measures: 1) NK cell lysis at five E:T ratios, 2) response of NK cells to rIFN γ and rIL-2 stimulation at five E:T ratios each, and 3) the PBL blastogenic response to PHA and Con A and proliferative response to the T3 MAb at three concentrations or dilutions each.

We were interested in the role of stress in predicting these outcomes, over and above the impact of disease and recovery variables on the immune response. Thus, we chose to control for three variables: 1) age, which is associated with down-regulation of the immune system; 2) disease stage, which is an indicator of the extent or burden of disease; and 3) days since surgery, which is an indicator of the degree of recovery from surgical stress and related factors (e.g., anesthesia).

Using hierarchical multiple regression (29), we tested the predictive value of psychologic stress for the measured immune outcomes. This procedure enters variables in a specified sequence and, at the final step, provides a test of the variance of the dependent variable (immune outcome) due to the predictor (stress), above and beyond the contribution of the control variables (age, stage, and days since surgery). In these regression analyses, age, days since surgery, and IES were considered as numerical variables. Stage was a categorical variable with two levels: II versus III.

For all of the analyses described below, any missing data were managed by the pairwise deletion technique, wherein each bivariate association is estimated with the use of all subjects for whom measures on both variables are available. This approach allows for more complete usage of available data than do alternative procedures (e.g., listwise deletion). For all of the dependent variables except the response of NK cells to rIFN γ , the quantity of missing data was small—with never more than 10 observations missing for any bivariate association. Effective sample sizes for the regression analyses ranged from 113 for the NK cell lysis ratios to 103 for T3 MAb values. For rIFN γ measures, sample sizes varied from 85 to 49 across the range of concentrations employed.

For each analysis, we provided three regression models: models A, B, and C. Model A includes only the control (independent) variables (i.e., age, stage, and days since surgery) in predicting the immune outcome (e.g., NK cell lysis). Predictors in model A were introduced simultaneously because we had

no basis for or a strong interest in investigating their effects in any particular sequence. Model B includes the three control variables as well as the psychologic stress variable (IES) in the prediction of the immune outcome. Of particular interest in this analysis was the increment in the squared multiple correlation (R^2) from model A to model B (i.e., R^2_{H-1}), indicating variance in a dependent variable (e.g., NK cell lysis) attributable to stress (IES) beyond that explained by the control predictors. In addition, the standardized regression beta (β) for the psychologic stress variable (IES) in model B (i.e., β_{stress}) indicates the magnitude and direction of the influence of this predictor on the dependent variable. The significance of the β weight was also tested. Finally, model C indicates the contribution of psychologic stress as the lone predictor; this third model provides the simple association between psychologic stress and immune function.

Results

Analyses Predicting NK Cell Lysis

Table 1 provides the results from the three models, A, B, and C, predicting NK cell lysis. For model A, in which age, stage, and days since surgery are the independent variables, R^2_A was small and nonsignificant for every E:T ratio (all F ratios were <1.0). Because the percentage of NK cells available would influence the

Table 1. Results of regression analyses for predicting natural killer (NK) cell lysis across six effector-to-target cell (E:T) ratios

| | Dependent variable: NK cell lysis at E:T ratios | | | | | |
|-------------------------|---|--------|--------|--------|--------|---------|
| | 100:1 | 50:1 | 25:1 | 12.5:1 | 6.25:1 | 3.125:1 |
| Model A, R^2_A | .005 | .007 | .012 | .015 | .020 | .023 |
| Model AA, R^2_{AA} | .085 | .148 | .185 | .233 | .250 | .241 |
| Model B | | | | | | |
| R^2_B | .135 | .212 | .238 | .268 | .275 | .253 |
| R^2_{H-1} | .050 | .064 | .053 | .035 | .025 | .012 |
| β_{stress} | -.234 | -.265 | -.240 | -.194 | -.165 | -.115 |
| $ndf = 110$ | -2.462 | -2.921 | -2.672 | -2.223 | -1.892 | -1.280 |
| P | .016 | .004 | .008 | .028 | .062 | .204 |
| Model C | | | | | | |
| R^2_C | .067 | .091 | .084 | .066 | .056 | .032 |
| $ndf = 110$ | -2.826 | -3.338 | -3.199 | -2.811 | -2.558 | -1.867 |
| P | .006 | .002 | .002 | .006 | .012 | .066 |

*Model A includes the control predictors of age, stage, and days since surgery for the immune outcome, NK cell lysis. The R^2_A is the total variance in NK cell lysis explained by these three predictors.

†Model AA includes model A variables plus the control predictor percentage of NK cells for the immune outcome, NK cell lysis. The R^2_{AA} is the total variance in NK cell lysis explained by these four predictors.

‡Model B includes model AA control variables plus the stress predictor (i.e., Impact of Event Scale [IES] score) for the immune outcome, NK cell lysis. The R^2_B is the total variance in NK cell lysis explained by the four control predictors and the stress predictor.

§ R^2_{H-1} is the increment in variance due to stress only (i.e., variance beyond that explained by the control predictors) in predicting the NK cell lysis outcome.

|| β_{stress} is the standardized regression beta (β) for the stress variable in model B. It indicates the magnitude and direction of the influence (negative) of stress on the immune outcome.

¶ ndf refers to the degrees of freedom in model B.

#Model C includes stress as the only predictor of the immune outcome, NK cell lysis. The R^2_C is the total variance in NK cell lysis explained by stress; this model provides the simple association between psychologic stress and immune function.

total NK cell activity as measured by lysis, we next added the percentage of NK cells, as determined by flow cytometry, into the analyses as an additional, independent control variable as shown (model AA). Across all E:T ratios, the R^2_{AA} values suggested that this variable added significant variance, as predicted, yielding R^2_{AA} values ranging from .085 to .250.

More important was the addition of the stress variable (IES) as a predictor, shown in model B. The value of R^2_B for lysis was noticeably larger than that of R^2_{AA} , and it provided a significant increment in prediction across the E:T ratios. These data indicate that the measure of psychologic stress that was used accounted for significant variance in NK cell lysis above and beyond that explained by age, stage, days since surgery, and percentage of NK cells. Moreover, the sign of the β regression coefficient for IES was negative, as predicted, indicating that an increase in measured stress was associated with a decline in NK cell lysis. The t tests for these coefficients were significant at five of the six E:T ratios. Also, no other predictor in model B had a significant regression coefficient.

We also provide the regression results when only IES was used as a predictor, eliminating the control predictors from the model (model C in Table 1). These results showed that the simple association between IES and NK cell lysis was statistically significant at five of the six E:T ratios.

Analyses Predicting Response of NK Cells to Cytokines

Results for the NK cell response to rIFN γ are provided in Table 2 and show a similar pattern. For model A, which used age, stage, and days since surgery as the independent variables, the value of R^2_A was small to moderate, ranging from .025 to .138. When stress (IES) was added to the model B regression, the R^2 values were statistically significant at all but one E:T ratio (50:1). Furthermore, the increments in the prediction due to IES, R^2_{B-A} , were significant and ranged from .054 to .119. This value reflects the proportion of variance in the cell response accounted for by stress (IES) beyond that explained by the control variables. Again, the negative weight of β for IES in model B indicated a negative influence of psychologic stress on the response of the NK

Table 2. Results of regression analyses for predicting natural killer (NK) cell response to recombinant interferon gamma (rIFN γ) across five effector-to-target cell (E:T) ratios

| | Dependent variable: NK cell response to rIFN γ at E:T ratios | | | | |
|--------------------|---|--------|--------|--------|---------|
| | 50:1 | 25:1 | 12.5:1 | 6.25:1 | 3.125:1 |
| Model A, R^2_A * | .025 | .097 | .080 | .138 | .124 |
| Model B† | | | | | |
| R^2_B | .041 | .151 | .197 | .257 | .208 |
| R^2_{B-A} ‡ | .016 | .054 | .117 | .119 | .084 |
| β_{Stress} § | -.128 | -.244 | -.358 | -.358 | -.301 |
| t | -1.104 | -2.190 | -3.203 | -3.084 | -2.083 |
| $df $ | 82 | 81 | 74 | 65 | 46 |
| P | .274 | .032 | .002 | .004 | .044 |
| Model C¶ | | | | | |
| R^2_C | .015 | .077 | .149 | .149 | .088 |
| t | -1.128 | -2.586 | -3.581 | -3.343 | -2.080 |
| $df $ | 82 | 81 | 74 | 65 | 46 |
| P | .264 | .012 | .002 | .002 | .044 |

*Model A includes the control predictors of age, stage, and days since surgery for the immune outcome, NK cell response. The R^2_A is the total variance in NK cell response explained by these three predictors.

†Model B includes model A control variables plus the stress predictor (i.e., Impact of Event Scale [IES] score) for the immune outcome, NK cell response. The R^2_B is the total variance in NK cell response explained by the three control predictors and the stress predictor.

‡ R^2_{B-A} is the increment in variance due to stress only (i.e., variance beyond that explained by the control predictors) in predicting the NK cell response.

§ β_{Stress} is the standardized regression beta (β) for the stress variable in model B. It indicates the magnitude and direction of the influence (negative) of stress on the immune outcome.

|| df refers to the degrees of freedom in model B.

¶Model C includes stress as the only predictor of the immune outcome, NK cell response. The R^2_C is the total variance in NK cell response explained by stress; this model provides the simple association between psychologic stress and immune function.

cells to rIFN γ . Again, no other predictor in model B had a significant regression coefficient. Finally, the results for model C in Table 2 showed a simple association between IES and the rIFN γ response. These correlations were significant at four of the five E:T ratios; the proportions of variance accounted for were in the range of .077 to .149.

We attempted to calculate a parallel set of regressions for the response of NK cells to rIL-2. However, cells from a large proportion of the patients (62%) had no response to rIL-2. When the regressions were conducted on data obtained from the remaining patients (38%), the addition of stress (IES) in model B produced a significant R^2 value at the 25:1 E:T ratio only. It appeared that the majority of the subjects' NK cells did not respond to treatment with rIL-2.

Analyses Predicting Blastogenic Response of PBLs to Con A, PHA, and the T3 MAh

Table 3 shows regression results for the Con A and PHA blastogenic responses across three concentrations each. Because the findings are similar for both assays, they will be discussed together.

For model A, which used age, stage, and days since surgery as the independent variables, the value of R^2_A for Con A ranged from .035 to .054 and was of similar magnitude for PHA, ranging from .022 to .033. Since the number of total T cells available will affect the blastogenesis values, we next added the number of T3-positive cells into the analyses as an additional, independent control variable as shown by the step model AA. Across all concentrations for each mitogen, the value of R^2_{AA} suggested that this variable added variance, yielding the R^2_{AA} values ranging from .105 to .125 for Con A and from .023 to .033 for PHA.

The addition of stress (IES) to the regression for blastogenesis added significant variance, as indicated in model B. All of the R^2 values were statistically significant. Considering the increments in R^2 due to stress (IES), these were significant and ranged from .032 to .061 for Con A and from .047 to .060 for PHA, reflecting the proportion of variance in the blastogenesis accounted for by IES beyond that explained by the control variables. Again, the negative β weights for IES in model B indicated a negative influence of psychologic stress on the blastogenic responses

Table 3. Results of regression analyses for predicting the blastogenic response to concanavalin A (Con A) and phytohemagglutinin A (PHA) across three concentrations each

| | Dependent variable: blastogenic response of mitogen | | | | | |
|-------------------------|---|------------|--------------|-------------|------------|--------------|
| | Con A | | | PHA | | |
| | 10 μg/mL | 5 μg/mL | 2.5 μg/mL | 10 μg/mL | 5 μg/mL | 2.5 μg/mL |
| Model A. R^2_A * | .035 | .043 | .054 | .022 | .024 | .033 |
| Model AA. R^2_{AA} † | .105 | .125 | .115 | .023 | .024 | .033 |
| Model B‡ | | | | | | |
| R^2_B | .166 | .174 | .147 | .083 | .074 | .080 |
| R^2_{B-AA} § | .061 | .049 | .032 | .060 | .050 | .047 |
| β_{stress} | -.255 | -.229 | -.187 | -.256 | -.234 | -.229 |
| $n(df = 103)$ ¶ | -2.668 | -2.401 | -1.927 | -2.521 | -2.299 | -2.254 |
| P | .010 | .018 | .058 | .014 | .024 | .026 |
| Model C# | | | | | | |
| R^2_C | .053 | .065 | .053 | .070 | .054 | .052 |
| $n(df = 108)$ ¶ | -2.443 | -2.724 | -2.443 | -2.857 | -2.489 | -2.441 |
| P | .016 | .008 | .016 | .006 | .014 | .016 |

*Model A includes the control predictors of age, stage, and days since surgery for the immune outcome, blastogenesis. The R^2_A is the total variance in blastogenesis explained by these three predictors.

†Model AA includes model A variables plus the control predictor of number of T cells for the immune outcome, blastogenesis. The R^2_{AA} is the total variance in blastogenesis explained by these four predictors.

‡Model B includes model AA control variables plus the stress predictor (i.e., Impact of Event Scale [IES] score) for the immune outcome, blastogenesis. The R^2_B is the total variance in blastogenesis explained by the four control predictors and the stress predictor.

§ R^2_{B-AA} is the increment in variance due to stress only (i.e., variance beyond that explained by the control predictors) in predicting the blastogenesis outcome.

|| β_{stress} is the standardized regression beta (β) for the stress variable in model B. It indicates the magnitude and direction of the influence (negative) of stress on the immune outcome.

¶ df refers to the degrees of freedom in model B.

#Model C includes stress as the only predictor of the immune outcome, blastogenesis. The R^2_C is the total variance in blastogenesis explained by stress; this model provides the simple association between psychologic stress and immune function.

across concentrations. Moreover, no other predictor in model B had a significant regression coefficient. Finally, results for model C in Table 3 showed a simple association between stress (IES) and the blastogenic response. These correlations were significant for each concentration of Con A and PHA.

Table 4 shows regression results for the proliferative response of T cells to three different dilutions of the T3 MAb. For model A, the control R^2 values were not significant for any dilution. Addition of number of T3-positive cells available as a control increased the variance accounted for as shown by the step model AA. The R^2_{AA} values ranged from .088 to .143. However, increments in R^2 due to the addition of stress (IES), as shown by R^2_{B-AA} , were significant, ranging from .056 to .067. This indicates that about 6% of the variance was accounted for by stress (IES) beyond that explained by the control variables. Once again, no other predictor in model B had a significant regression coefficient. Results for model C again showed the simple, significant as-

sociation of stress (IES) with the response to the T3 MAb at all dilutions, with R^2_C values of .092 to .102.

Discussion

Any immune response involves a complex cascade of events that occur over time. Studies suggest that the peripheral products of stress can play numerous roles in regulating immunity, and so the effects of stress will, necessarily, be variable. Current research suggests, for example, that the acute stressors, both real stressors [e.g., parachute jumps (30)] and artificial stressors [e.g., experimental tasks including speech or math stress (31)], are correlated with the mobilization (increase) of NK cells. These changes are thought to be a result of alterations in cell trafficking. In contrast, studies of chronic stressors [e.g., bereavement, caregiving, or divorce (7,9)] suggest that stress can have an effect on the ability of NK cells to lyse a target cell, the ability of NK cells to respond to rIFN γ and rIL-2 *in vitro*, and other aspects of the cellular immune response.

Our results suggest that stress, as assessed via a self-report measure of intrusive and avoidant thoughts and behaviors about cancer, was related to a negative effect on NK cell lysis, the ability of NK cells to respond to two cytokines, the blastogenic response of PBLs to two mitogens, and the proliferative response to MAb T-cell receptor. These effects were inhibitory and of similar magnitude (i.e., reliable), both between the assays and within an assay (i.e., across E:T ratios and mitogen concentrations). The analyses controlled for variables that might also be expected to exert short-term or long-term effects on immunity—such as age, stage of disease, and days since surgery—and ruled out other potentially confounding variables (e.g., nutritional status) that might also be influential. These controls reduced the plausibility of alternative, rival hypotheses for these consistent findings.

It is recognized that NK cells mediate natural immunity, but some researchers (32) suggest that their role in health generally has been underestimated. For example, there is evidence to suggest that the NK cells participate either directly or indirectly in multiple developmental, regulatory, and communication networks of the immune system. Furthermore, NK cells are efficient effector cells that not only are equipped for cell killing, but also are capable of rapid responses to exogenous or endogenous signals by producing cytokines and other factors involved in interactions between immune and non-immune cells (20).

The ability to spontaneously lyse a broad range of infected cells or tumor cells is the best known functional attribute of NK cells (20,22). Consistent with previous reports, these data suggest that stress may impair this important process. Our findings highlight the specific effect of cancer stress on immune function, whereas prior data obtained by Levy et al. (33) had suggested that women's reports of fatigue were related to lower levels of NK cell lysis. Chronically low levels of NK cell activity occur in patients with cancer, particularly when there are large tumor burdens or disseminated metastases (32). In general, patients with low NK cell activity appear to be at higher risk for infections, to have more prolonged diseases, or to suffer more severe symptoms

Table 4. Results of regression analyses for predicting proliferative response of peripheral blood leukocytes to a monoclonal antibody to T-cell receptor (T3) across three dilutions

| | Dependent variable: proliferative response at dilutions | | |
|------------------------|---|--------|--------|
| | 128:1 | 64:1 | 32:1 |
| Model A. R^2_A * | .026 | .052 | .064 |
| Model AA. R^2_{AA} † | .088 | .104 | .143 |
| Model B‡ | | | |
| R^2_B | .155 | .160 | .200 |
| R^2_{B-AA} § | .067 | .056 | .057 |
| β_{Stress} | -.273 | -.249 | -.252 |
| $t(df = 101)$ ¶ | -2.747 | -2.514 | -2.604 |
| P | .008 | .014 | .012 |
| Model C# | | | |
| R^2_C | .102 | .092 | .094 |
| $t(df = 101)$ ¶ | -3.452 | -3.255 | -3.307 |
| P | .002 | .002 | .002 |

*Model A includes the control predictors of age, stage, and days since surgery for the immune outcome, proliferative response. The R^2_A is the total variance in proliferation explained by these three predictors.

†Model AA includes model A variables plus the control predictor of number of T cells for the immune outcome, proliferation. The R^2_{AA} is the total variance in proliferation explained by these four predictors.

‡Model B includes model AA control variables plus the stress predictor (i.e., Impact of Event Scale [IES] score) for the immune outcome, proliferation. The R^2_B is the total variance in proliferation explained by the four control predictors and the stress predictor.

§ R^2_{B-AA} is the increment in variance due to stress only (i.e., variance beyond that explained by the control predictors) in predicting the proliferation outcome.

|| β_{Stress} is the standardized beta (β) for the stress variable in model B. It indicates the magnitude and direction of the influence (negative) of stress on the immune outcome.

¶df refers to the degrees of freedom in model B.

#Model C includes stress as the only predictor of the immune outcome, proliferation. The R^2_C is the total variance in proliferation explained by stress; this model provides the simple association between psychologic stress and immune function.

than patients whose NK cell activity remains normal (32,34).

A variety of biologic response modifiers are known to increase the activation, proliferation, or cytotoxicity of NK cells (20). Among the best known activators of NK cells are IL-2 and IFN γ . Our data show that the physiologic changes associated with psychologic stress inhibited NK cell lysis. Stress also affected the ability of NK cells to respond to rIL γ , a finding that is consistent with two previous reports involving another life stressor [i.e., caregiving for a spouse with Alzheimer's disease (9,26)]. It is interesting that NK cells from 62% of the women did not respond to rIL-2. In subsequent analyses comparing women who did have an rIL-2 response with those who did not, no stress or disease variable differentiated the two groups. Further studies will need to be performed to explore this result, although it is possible that the lack of responsiveness of NK cells to rIL-2 may be due to an overproduction of prostaglandin E_2 by monocytes. It has been suggested that in breast cancer patients prostaglandin E_2 decreases IL-2 production in effector cell populations, resulting in the down-

regulation of the expression of the IL-2 receptor on NK cells (23). Follow-up studies will need to pursue and clarify this difference in cytokine responses.

It has been shown that the ability of PBLs to respond to PHA is reduced, in general, in cancer patients (35); this lowered response is related to tumor burden and declines in the ability of PBLs to respond to PHA with disease progression (36). The negative effect of stress on blastogenesis was replicated in this study across two mitogens, PHA and Con A, as well as in the response of T cells to an MAb against the T-cell receptor. These findings are consistent with correlational and experimental studies indicating that stress impairs the blastogenic response of PBLs to mitogens and virus-specific T-cell responses (8,10,37-39). Mitogen-induced proliferation has been used to indicate the immune system's ability to respond to antigens from pathogens. Chronically stressed, but healthy, individuals showing decrements in the cellular immune response (including NK cell lysis and the response of the PBLs to mitogens) subsequently reported a higher incidence of infectious illnesses (8). If this

effect is reliable, these data would suggest that cancer patients who experience high levels of stress, lowered levels of responsive T lymphocytes, and decreased NK cell function may be at greater risk for infectious illnesses as they begin adjuvant therapy.

It is interesting that evidence is accumulating to suggest that psychologic and/or behavioral stress reduction interventions may enhance certain aspects of the cellular immune response, including NK cell lysis. In an early investigation, Kiecolt-Glaser et al. (40) studied 61 healthy adults living in a retirement home. After receiving 1 month of training in progressive muscle relaxation, the subjects showed evidence of a 30% increase in NK cell lysis in comparison with those who received no treatment or only social contact. Fawzy et al. (41) studied 61 patients with melanoma and reported that, 6 months after treatment, subjects receiving intervention had significantly higher levels of IFN alfa-augmented NK cell activity than those who received no treatment. These data suggest that, if behavioral interventions can reduce stress and enhance the cellular immune response, then health outcomes might improve.

In conclusion, these data show a down-regulation of different aspects of the cellular immune response associated with the psychologic stress that accompanies the diagnosis and initial surgical treatment of cancer. We note that these study participants are part of a larger effort testing the biobehavioral aspects of stress, immunity, and disease course (5). It will be important to document the longitudinal nature of these findings, and future studies will provide such data. Moreover, half of the women who participated have been randomly assigned to receive a psychologic/behavioral intervention specifically designed to reduce stress, enhance quality of life, and test for the biologic mechanism—such as immune responses—that may mediate any positive effects of stress reduction on health and disease outcomes.

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